

INTERACTIONS OF LOCAL ANESTHETICS AND CHOLINERGIC
LIGANDS WITH MEMBRANE-BOUND ACETYLCHOLINE
RECEPTOR FROM TORPEDO CALIFORNICA

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To Anne and my parents

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Abstract

A rapid method for preparation of membrane fractions highly enriched in nicotinic acetylcholine receptor from Torpedo californica electroplax is described in Chapter 1. This purification was achieved by sucrose-density-gradient centrifugation in a reorienting tube rotor. Further purification of these membranes was achieved by selective extraction of proteins at alkaline pH. The alkali treated membranes retained the functional properties of the untreated membranes and in addition contained essentially only the four polypeptides (molecular weights 40,000, 50,000, 60,000, and 65,000) characteristic of the receptor purified by affinity chromatography.

Chapter 2 describes the effects of local anesthetics on the membrane-bound acetylcholine receptor. The rate of the transition in receptor affinity (low to high) was determined by following the time-dependent increase in inhibition of iodinated α -bungarotoxin binding caused by 1 μ M carbamylcholine. At concentrations below those that directly inhibited the binding of iodinated α -bungarotoxin, dibucaine increased the rate of the transition to a high-affinity state and tetracaine decreased this rate. A parallel was observed between the effectiveness of a compound in increasing or decreasing the rate of the agonist-induced transition in affinity and the change in its apparent inhibition constant in the presence of carbamylcholine measured by the displacement of tritiated perhydrohistrionicotoxin. (Blanchard, S. G., Elliott, J., and Raftery, M. A. (1979) Biochemistry 18, 5880-5885). This parallel could be explained by assuming (a) that local anesthetics bound directly to the specific histrionicotoxin binding site

or (b) that they bound to a different site and the observed effects were caused indirectly by changes in receptor conformation.

The kinetics of local anesthetic binding to the receptor were examined using the fluorescent probe ethidium. The binding of tetracaine, dibucaine and lidocaine could not be described by a common mechanism and models which are qualitatively consistent with the data are discussed.

The preparation and characterization of the local anesthetic analog procaine amide azide are described in Chapter 3. When low concentrations of ^3H -labeled procaine amide azide were photolyzed in the presence of acetylcholine receptor containing membrane fragments, two major labeled bands with apparent molecular weights of $\sim 43,000$ and $90,000$ were seen after electrophoresis on sodium dodecyl sulfate/polyacrylamide gels. When the length of the gels was increased, the labeled band of lower molecular weight was resolved into a major protein of $43,000$ daltons and a minor one of $40,000$ daltons. The radioactivity incorporated into the $40,000$ dalton protein was due to [^3H] procaine amide azide binding to cholinergic ligand binding sites, whereas the $43,000$ and $90,000$ molecular weight bands appeared to be unrelated to the acetylcholine receptor. Experiments with alkali-treated membrane preparations showed that the $60,000$ and $65,000$ dalton receptor subunits were also labeled by procaine amide azide.

Chapter 4 describes the alkylation of acetylcholine receptor enriched membrane fragments (following their reduction with low concentrations of dithiothreitol) by the fluorescent probe 5-iodoacetamido salicylic acid. This modification did not affect the equilibrium binding

of carbamylcholine to the receptor. The fluorescence of bound 5-iodo-acetamido salicylic acid was enhanced when the labeled membrane fragments were mixed with carbamylcholine. This increase in fluorescence was abolished by preincubation of the membrane fragments with excess α -bungarotoxin and was therefore specific for the acetylcholine receptor. Estimates of dissociation constants obtained from centrifugation experiments using radioactive ligand and from fluorescence titration data were in good agreement, showing that the observed fluorescence enhancement was an accurate reflection of receptor-carbamylcholine complex formation. The kinetics of carbamylcholine binding to labeled membrane fragments have been investigated over a wide range of ligand concentrations using stopped-flow fluorescence techniques. The kinetic signal was complicated and four distinct exponential phases were observed. A kinetic mechanism has been proposed to account for this behavior.

Proposition Abstracts

Proposition I

The nicotinic acetylcholine receptor from Torpedo californica may have multiple acetylcholine binding sites. It is proposed that a photoaffinity analog of acetylcholine, diazoacetylcholine, be used to label the acetylcholine binding sites(s).

Proposition II

The preparation of a photolabile derivative of the putative polypeptide neurotransmitter substance P is described. This analog may be used to identify the endogenous receptor for substance P present in mammalian brain.

Proposition III

The kinetics and mechanism of the enzyme dimethylallyl pyrophosphate: umbelliferone dimethylallyl transferase may be studied by means of fluorescence spectroscopy. The intrinsic fluorescence of the substrate umbelliferone would be used as the spectroscopic probe.

Proposition IV

A method is proposed for the purification of the low density lipoprotein receptor using antibodies directed against low density lipoprotein. The purified receptor may be used in studies of the mechanism of receptor-mediated endocytosis.

Proposition V

The interactions of the calmodulin-binding protein, calcineurin, with calmodulin and calcium may be studied by fluorescence and absorption spectroscopy. These proteins may be involved in the modulation of cellular cyclic adenosine monophosphate levels.

Abbreviations Used:

AcCh	acetylcholine
AcChR	acetylcholine receptor
α -BuTx	α -bungarotoxin
[125 I] α -BuTx	α -bungarotoxin labeled with 125 I
carb	carbamylcholine
DEAE	diethylamino ethyl
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Eth	ethidium
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HTX	histrionicotoxin
H ₁₂ -HTX	perhydrohistrionicotoxin
IAcNH ₂	iodoacetamide
IAS	5-iodoacetamidosalicylic acid
MBTA	4-(N-maleimido)benzyltrimethylammonium iodide
SDS	sodium dodecyl sulfate
<u>Torpedo</u> Ringers	250 mM NaCl, 5 mM KCl, 2mM MgCl ₂ , 4 mM CaCl ₂ buffered by 20 mM Hepes or Tris (pH 7.4)
Tris	tris-(hydroxymethyl) amino methane

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INTRODUCTION

1. General

The transmission of a nerve impulse across the synapse at the neuromuscular junction is mediated by the chemical transmitter acetylcholine (AcCh). After its release from presynaptic nerve terminals, AcCh diffuses across the synapse and binds to specific receptors in the postsynaptic membrane (Katz, 1966). This combination of AcCh with acetylcholine receptors (AcChR) results in a transient increase in the permeability of the postsynaptic membrane for Na^+ and K^+ ions (Takeuchi and Takeuchi, 1959, 1960) triggering an action potential and, ultimately, muscle contraction.

Localization of the AcChR to the endplate regions of the postsynaptic membrane has been demonstrated using radioactively labeled (Barnard et al., 1971; Berg et al., 1972) and fluorescently labeled (Barrantes et al., 1975) α -bungarotoxin (α -BuTx). The postsynaptic membrane is highly folded at the synapse and acetylcholinesterase activity is evenly distributed along the folds (Salpeter et al., 1972). The AcChR, however, is concentrated at the crests of the synaptic folds where it reaches a density of 20,000-25,000/ μm^2 (Barnard et al., 1975).

The electric organs of marine elasmobranchs of the family Torpedinidae and of the fresh-water electric eel Electrophorus electricus are widely used as sources for the isolation and characterization of the AcChR. The electric organs consist of stacks of large, flattened cells which are synchronously discharged to generate voltage spikes. The receptor is found only on the (heavily) innervated face

of the electroplaques while the uninnervated face is inexcitable and has low resistance (Bennett, 1970). The electric eel exhibits both electrical and chemical excitability (Bennett, 1970) while Torpedo species show only chemical excitability (Bennett et al., 1961).

In Torpedo and Narcine electroplaques most of the innervated membrane surface is covered by synaptic contacts (Bennett et al., 1961; Bennett and Grundfest, 1961; Miledi et al., 1971) while in Electrophorus electricus only 1-2% of the surface of the plasma membrane is covered (Bourgeois et al., 1972). Torpedo is therefore a richer source of AcChR than is the electric organ of the electric eel. The electroplaques from Torpedo and Narcine are only 10-20 μ m in thickness (Matthewson et al., 1958; Bennett et al., 1961) making it difficult to obtain viable cells for electrophysiological study. Therefore, most electrophysiological studies on electric tissue have used Electrophorus electricus. A single electroplaque from this species may be isolated and mounted between two buffer-containing chambers. Since the uninnervated face of the cell has a low resistance, the electrical response to bath-applied drugs may be measured using extracellular electrodes (Schoeffeniels and Nachmansohn, 1957).

2. The Postsynaptically Acting Snake Toxins

The study of the AcChR has been greatly aided by the discovery that certain polypeptide toxins present in the venoms of snakes of the families Elapidae and Hydrophidae bind specifically and with high affinity to AcChR (Lee and Chang, 1966; Lee et al., 1967). These toxins are basic polypeptides which may be subdivided into "short" and

'long' classes. The short neurotoxins contain 61-62 amino acids and four disulfide bonds while the long toxins contain 71-74 amino acid residues and five disulfide bridges (Lee, 1972). Examples of the two classes are cobratoxin from Naja naja atra with 62 residues and α -BuTx with 74 amino acid residues (Mebs et al., 1972). These neurotoxins form a non-covalent complex with AcChR which is slowly reversible in the case of the short toxins (Su et al., 1967; Tarzieff-Depierre and Pierre, 1966) but essentially irreversible for the long neurotoxins (Chang and Lee, 1963; Lee and Chang, 1966; Changeux et al., 1970; Miledi et al., 1971). In the case of α -BuTx the association rate, assuming a simple bimolecular reaction, is $\sim 1.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ while the lifetime of the complex is longer than two days (Blanchard et al., 1979b). If one assumes, therefore, that $< 10\%$ of the bound α -BuTx dissociates from the AcChR in two days, the rate of dissociation may be calculated to be $< 6 \times 10^{-7} \text{ s}^{-1}$ and the apparent dissociation constant is $< 4 \times 10^{-11} \text{ M}$.

The reasons for this extremely tight binding are not understood. However, analysis of the three-dimensional structure of erabutoxin a from the sea snake Laticanda semifasciata showed the existence of an extended loop containing most the amino acid residues essential for activity (Tsernoglou and Petsko, 1976, 1977). The authors concluded that, on binding, this loop was inserted into a corresponding cleft on the AcChR.

Cholinergic ligands inhibit toxin binding to the solubilized, purified (Moody et al., 1973; Schmidt and Raftery, 1974; Meunier et al., 1974;

Maelicke et al., 1977) and membrane-bound AcChR (Weber and Changeux, 1974; Bulger et al., 1977; Quast et al., 1978b). Although the underlying molecular mechanism of this inhibition is unknown (Quast et al., 1978b), the inhibition as a function of ligand concentration is often reasonably well described by simple hyperbolae from which inhibition constants K_i can be determined (Weber and Changeux, 1974; Vandlen et al., 1976). These inhibition constants show good agreement with equilibrium constants for the interaction of ligand with AcChR as determined by other methods.

3. Preparation and Characterization of Detergent-Solubilized and Membrane-Bound AcChR

The AcChR was initially isolated as a detergent solubilized complex with [125 I] or [3 H] labeled α -toxin (Miledi et al., 1971; Raftery et al., 1971; Clark et al., 1972; Eldefrawi and Eldefrawi, 1972). Shortly thereafter, affinity chromatography was used to prepare the free solubilized receptor from Narcine (Schmidt and Raftery, 1972), Torpedo californica (Raftery, 1973; Schmidt and Raftery, 1973; Weill et al., 1974), T. marmorata (Karlsson et al., 1973; Eldefrawi and Eldefrawi, 1973), and T. nobiliana (Ong and Brady, 1974) as well as from Electrophorus electricus (Biesecker, 1973; Klett et al., 1973; Chang, 1974; Lindstrom and Patrick, 1974; Meunier et al., 1974). Although initial reports of subunit composition varied widely, a major band of $\sim 40,000$ molecular weight was found in most cases. These differences in apparent subunit composition have been attributed to proteolytic degradation of the receptor (Vandlen et al., 1976). When

EDTA is added to the preparation to inhibit calcium-activated proteases (H.-P. H. Moore and M. A. Raftery, unpublished) and phospholipases (W. Wu and M. A. Raftery, unpublished) a constant pattern is seen on SDS-polyacrylamide gel electrophoresis.

The purified AcChR consists of four polypeptide chains of 40,000, 50,000, 60,000, and 65,000 daltons (Raftery et al., 1974; Weill et al., 1974; Vandlen et al., 1976) with an apparent stoichiometry of 2:1:1:1 as determined by N-terminal amino acid analysis (Strader et al., 1980a). The molecular weight of $\sim 255,000$ calculated from this subunit ratio is in good agreement with values of $\sim 250,000$ - $275,000$ obtained by sedimentation experiments (Raftery, et al., 1972, 1974; Meunier et al., 1972; Reynolds and Karlin, 1978), electrophoresis of crosslinked AcChR (Biesecker, 1973; Hucho and Changeux, 1973), and osmometry (Martinez-Carrion et al., 1975).

In detergent solution the AcChR exists as both $\sim 9S$ and $\sim 13S$ species (Raftery et al., 1972) with similar ligand binding properties (Raftery et al., 1972; Sugiyama and Changeux, 1975; Gibson et al., 1976). The 13S species has been shown to be a dimer of the 9S form covalently linked by a disulfide bond between the 65,000 dalton subunits (Chang and Bock, 1977; Hamilton et al., 1977; Witzemann and Raftery, 1978a; Hucho et al., 1978).

The solubilized, purified receptor is a glycoprotein containing carbohydrate on all four subunits. The predominant neutral sugar is mannose with lesser amounts of glucose and galactose present (Vandlen et al., 1979). Phosphorylation of the AcChR has also been reported (Gordon et al., 1977; Tefehberg et al., 1977) although its importance

in receptor function is unknown. Phosphoserine is the only phosphorylation product detected in purified receptor. There are about seven phosphoserines per AcChR monomer and all subunits are phosphorylated (Vandlen *et al.*, 1979).

Membrane fragments enriched in AcChR have been prepared by sucrose density gradient centrifugation of homogenates of electric tissue from T. California (Duguid and Raftery, 1973; Reed *et al.*, 1975). SDS-polyacrylamide gel electrophoresis of these preparations shows major bands corresponding to those seen with the purified AcChR as well as components of 43,000 and 90,000 daltons. One preparation consisting of primarily 40,000 and 43,000 dalton polypeptides has been described for T. marmorata (Sobel *et al.*, 1977). However, this pattern difference must arise from the different preparative procedures used in different laboratories since the AcChR's from different species showed similar polypeptide compositions when purified using one method (Deutch and Raftery, 1979). The 43,000 dalton component is not actin since actin from T. californica migrates with an apparent molecular weight of 47,000 on SDS-polyacrylamide gels (Strader *et al.*, 1980b). Recently, the use of improved homogenization techniques coupled with centrifugation in a vertical tube rotor has allowed rapid preparation of membrane fragments of high specific activity and yield (Chapter 1; see also Elliott *et al.*, 1980). In addition, the treatment of AcChR containing membrane fragments with base as described for erythrocyte ghosts (Steck and Yu, 1973) results in functional preparations that contain essentially only the four polypeptides characteristic of the purified receptor (Neubig *et al.*, 1979; Elliott *et al.*, 1980; Chapter 1).

Electron microscopy of AcChR membrane preparations shows the presence of rosettes 80-90 Å in diameter with an electron-dense center "pit" (Nickel and Potter, 1973; Cartaud et al., 1973; Raftery et al., 1974). These structures have recently been identified with the AcChR (Klymkowsky and Stroud, 1979). Low-angle X-ray diffraction studies of AcChR membranes from T. californica indicated that the protein spanned the lipid bilayer, extending 55 Å on one side but only 15 Å on the other (Ross et al., 1977). Hartig and Raftery (1977) using lactoperoxidase catalyzed labeling with ¹²⁵I found that in membranes the 40,000, 50,000, and 60,000 dalton subunits were labeled and therefore exposed to solvent while the (apparently buried) 65,000 subunit was not labeled. Recently Strader et al. (1979) have used a preparation consisting of sealed membrane vesicles (Hartig and Raftery, 1979) and anti-AcChR antibodies to demonstrate the trans-membrane nature of the receptor.

4. Equilibrium Ligand Binding Properties of AcChR

The direct binding of various radiolabeled cholinergic ligands to the AcChR has been studied by use of equilibrium dialysis in the case of solubilized, purified receptor (Moody et al., 1973; Klett et al., 1973; Eldefrawi and Eldefrawi, 1973; Meunier et al., 1974) and by centrifugation assay for the membrane-bound receptor (Raftery et al., 1975; Quast et al., 1978a; Neubig and Cohen, 1979). Inhibition constants for ligand binding to membrane-bound AcChR have also been determined from the inhibition of the rate of radiolabeled α -toxin binding as a function of ligand concentration (Weber and Changeux, 1974; Vandlen

et al., 1976; Blanchard et al., 1979b). In the case of solubilized, purified receptor, however, the interaction with toxin is complex so that the determination of K_I is dependent on the choice of a model for toxin binding (Blanchard et al., 1979b).

A number of photoaffinity analogs of cholinergic ligands have been used to determine which AcChR subunits are involved in ligand binding. Hucho et al. (1976) used 4-azido-2-nitrobenzyltrimethylammonium fluoroborate to label solubilized, purified receptor. All four subunits were labeled but α -toxin protected only the 40,000 dalton subunit from labeling. A bis-azido derivative of the antagonist DAP has also been used to label both membrane-bound and solubilized AcChR from T. californica. The 40,000 dalton subunit was labeled in both cases. However, the 50,000 dalton subunit was also labeled in membranes while the 60,000 dalton polypeptide was labeled in the purified receptor (Witzemann and Raftery, 1977). In both solubilized and membrane-bound preparations α -BuTx protected against labeling of both the 40,000 and the secondary (50,000 or 60,000 dalton, respectively) labeled bands. In both of the above studies the non-40,000 labeling was postulated to be due to diffusion of reactive nitrene from the main binding site on the 40,000 dalton polypeptide to nearby subunits where reaction with appropriately situated nucleophiles occurred. Although this interpretation cannot be ruled out, the recent discovery that the 40,000, 50,000, 60,000, and 65,000 dalton subunits are homologous polypeptides (Raftery et al., 1980) suggests that the non-40,000 labeling may be the result of specific binding to secondary sites present on the other subunits.

Ethidium bromide has been used as a fluorescent probe of ligand binding to the AcChR (see below). Therefore, bis-azido ethidium chloride was used to determine the site(s) of ethidium interaction (Witzemann and Raftery, 1978b). The 40,000 dalton subunit was the major labeled band. In the presence of carb, however, there was increased labeling in the 50,000, 60,000, and (to a lesser extent) 40,000 dalton bands. The increased labeling was probably a reflection of intersubunit conformational effects (Witzemann and Raftery, 1978b).

The 40,000 dalton polypeptide was directly demonstrated to bind α -toxin by use of a photoaffinity derivative of α -BuTx (Witzemann et al., 1979). The 65,000 dalton subunit was also labeled. In addition, when a long spacer arm was incorporated between the toxin and the azido functionality only the 65,000 dalton polypeptide was labeled indicating that the 40,000 and 65,000 dalton components were in close proximity in the membrane. Because α -BuTx binds to the AcChR on the outside of the membrane these studies also showed that the 65,000 dalton polypeptide was exposed to the aqueous phase on the outside face of the membrane.

Another approach to affinity labeling of the AcChR made use of the discovery (Karlin, 1969; Ben Haim et al., 1973) of a readily reducible disulfide bond in the region of the acetylcholine binding site. Both the cholinergic antagonist 4-(N-maleimido)benzyltrimethylammonium iodide (Karlin and Cowburn, 1973; Weill et al., 1974) and the agonist bromo-acetylcholine Chang et al., 1977; Damle et al., 1978; Moore and Raftery, 1979a) labeled only the 40,000 dalton polypeptide.

5. Stoichiometry of Ligand Binding

There is little agreement in the literature concerning the stoichiometry of α -BuTx sites to high affinity agonist sites. Values of 2:1 for the solubilized, purified (Moody et al., 1973; Maelicke et al., 1977) and the membrane-bound receptor (Rafferty et al., 1975; Quast et al., 1978a; Schimerlik et al., 1979a) have been reported by some groups while other workers have found this ratio to be 1:1 (Weber and Changeux, 1974; Sugiyama and Changeux, 1975; Damle et al., 1976; Neubig and Cohen, 1979). These differences are not due to errors in the specific activities of the radiochemicals used since the same laboratory has found ratios of both 2:1 (Rafferty et al., 1975; Quast et al., 1978a; Schimerlik et al., 1979a) and 1:1 (Dunn et al., 1980; Chapter 1) using the same [^3H]-carb and [^{125}I] α -BuTx that was calibrated using the same unlabeled α -BuTx standard. Thus, under some (unknown) conditions it is possible to change the ratio of α -toxin to carb sites. The available evidence suggests that this change in ligand binding stoichiometry from 2:1 to 1:1 (toxin:agonist) is the result of a loss of toxin sites rather than a gain in agonist binding sites. For instance, preparations which show a 1:1 ratio of α -toxin:carb binding have an α toxin:HTX stoichiometry of $\sim 1:0.35$ (Elliott et al., 1979, 1980) whereas 2:1 α -toxin:carb membranes have an α -toxin:HTX stoichiometry of 4:1 (Elliott and Rafferty, 1977). Since carb and HTX bind to different sites this suggests that the number of α -BuTx binding sites has changed. In addition when membranes that initially show 2:1 stoichiometry are reduced with DTT and then alkylated with IAcNH₂ to "lock" them in a

low-affinity state (Moore and Raftery, 1979b) only 50% of the initial toxin binding sites are recovered. However, no loss of toxin binding occurs when the same treatment is carried out on membranes initially of 1:1 stoichiometry (S. M. J. Dunn and M. A. Raftery, unpublished).

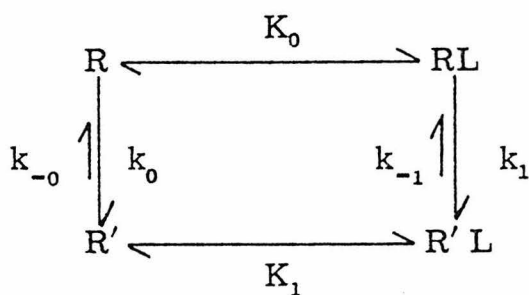
This change in the apparent number of α -BuTx sites may explain why alkali treated membrane preparations which contain only the four receptor subunits (as shown by SDS-polyacrylamide gel electrophoresis) have maximal specific activities approaching only 5 nmoles/mg protein (Neubig et al., 1979; Elliott et al., 1980) while the specific activity of pure receptor is calculated to be 8 nmoles/mg (as calculated from a molecular weight of 250,000).

Recently, the purification and characterization of a "long" type neurotoxin from the venom of the green mamba Dendroaspis viridis has been reported (Hanley et al., 1979; Patrick et al., 1980). This toxin was found to bind to twice as many sites on membrane-bound receptor as does α -BuTx. However, the ratio of mambatoxin: α -BuTx sites was reduced to 1:1 on solubilization with the non-ionic detergent Triton X-100 due to a loss of mambatoxin binding sites (Patrick et al., 1980). Unfortunately, the stoichiometry of α -BuTx and carb sites was not reported.

6. Transitions in Ligand Affinity

Prolonged application of agonists at the neuromuscular junction results in a decreased effectiveness of agonist in producing depolarization (Katz and Thesleff, 1957). These authors termed this phenomenon "desensitization" and explained the observations in terms of a two-state

model in which receptor, initially in a low ligand affinity state was changed to a high ligand affinity state by the action of agonist. This model may be written as:



where R and R' are the low and high affinity states of the receptor, respectively and L is ligand. K_0 and K_1 are dissociation constants for ligand binding while the k_i are rate constants for the receptor isomerization. Rang and Ritter (1969, 1970a, b) found that preincubation with agonists caused an increase in affinity for some antagonists and that this affinity change was independent of the ligand used to induce this "metaphilic effect". Desensitization has also been observed in electrophysiological preparations from Torpedo nobiliana (Bennett et al., 1961) and T. marmorata (Moreau and Changeux, 1976).

Changes in ligand affinity toward membrane-bound AcChR as a function of incubation time with ligand have been detected by the inhibition of toxin binding (Weber et al., 1975; Lee et al., 1977) and also using a spin-labeled agonist (Weiland et al., 1976). This affinity change is considered to be an in vitro correlate to the electrophysiological phenomenon of desensitization and the observations have been qualitatively explained (Weber et al., 1975; Weiland et al., 1976; Colquhoun and Rang, 1976; Lee et al., 1977) in terms of the two-state model originally

proposed by Katz and Thesleff (1957).

Quantitative treatment of the rate and equilibrium constants for carb binding (as determined by inhibition of [125 I] α -BuTx binding) revealed that the two-state model could not adequately account for the data (Quast et al., 1978b). In particular, the data indicated that only about 50% of the AcChR should be in the low affinity form while the experimental observations showed that $>90\%$ of the receptor initially bound ligand with low affinity. Other workers, however, have reported that their data can be fit by the two-state model (Weiland et al., 1977). Although these differences have not been resolved it should be noted that the buffers of Weiland et al. did not contain calcium which affects both pharmacological desensitization (Magazanik, 1976) and the equilibrium binding of AcCh (Weber and Changeux, 1974).

Ligand-induced conformational changes in AcChR have also been detected using fluorescence stopped-flow methods. The intrinsic fluorescence of membrane-bound AcChR has been used to monitor the effects of agonist binding (Bonner et al., 1976; Barrantes, 1976). A slow conformational change was observed which these authors attributed to desensitization. The use of an extrinsic fluorescent probe such as the antimalarial, quinacrine (Grünhagen and Changeux, 1976; Grünhagen et al., 1976, 1977) gave a larger signal than is available by intrinsic fluorescence. Two kinetic processes were seen on addition of agonist to quinacrine-labeled membrane fragments: a fast fluorescence increase in the millisecond time range which was identified with receptor activation (Grünhagen et al., 1977b) followed by a slower increase attributed to desensitization (Grünhagen and Changeux, 1976;

Grünhagen et al., 1977a,b). However, quinacrine has been shown to act as a local anaesthetic (Grünhagen and Changeux, 1976) and the kinetics of the fast phase are very similar to the blocking effect of quinacrine at the neuromuscular junction (Adams and Feltz, 1977) suggesting that quinacrine seriously altered receptor kinetics.

Another extrinsic probe, ethidium bromide (Schimerlik and Raftery, 1976; Schimerlik et al., 1979a,b; Quast et al., 1978a) has been used to study ligand binding to the AcChR and does not act as a local anesthetic at the concentrations used (Schimerlik et al., 1979a). The carb binding data could not be fit by a simple two-state model but could be described by a model which involves sequential ligand binding (Quast et al., 1978a, Quast et al., 1979; see also Chapter 4 for a discussion). The binding of two fluorescent acetylcholine analogs has also been described (Jürss et al., 1979; Heidmann et al., 1979). Chapter 4 details a technique for covalently introducing a fluorescent probe near the AcCh binding site which overcomes various disadvantages of the methods described above.

7. Assay of AcChR Function

The functional integrity of isolated AcChR membrane fragments may be determined by following the release of vesicle-entrapped $^{22}\text{Na}^+$ in response to cholinergic agonists. Initial studies using this method (Kasai and Changeux, 1971) reported a time course for efflux with a half-time of minutes whereas permeability changes in vivo occur on a time scale of a few milliseconds. Hess et al. (1978) reported a bi-phasic ion flux with a half time of seconds using membrane preparations

from Electrophorus electricus. Recently, the agonist-induced release of $^{22}\text{Na}^+$ has been found to be faster than the first timepoint obtainable by filtration assay (i.e., 10 sec) (Miller et al., 1978; Moore et al., 1979b). This method has been used to demonstrate that only the 40,000, 50,000, 60,000, and 65,000 dalton polypeptides are necessary for the AcChR function (Moore et al., 1979a).

Two methods have been described for the measurement of flux responses on a sub-second time scale. The first measures the amount of $^{86}\text{Rb}^+$ taken up by membrane vesicles after rapid mixing with carb to initiate the reaction followed by quenching of the flux response by addition of excess d-Tubocurarine (d-TC) at various times after mixing (Hess et al., 1979; Cash and Hess, 1980). This method suffers from a limited time resolution of ~ 25 msec per point. The second method (Moore and Raftery, 1980) monitors quenching of a vesicle entrapped fluorophore by thallium(I). The resulting fluorescence decrease can be monitored by conventional stopped-flow techniques with a typical mixing time of 2-3 msec.

8. Functional Reconstitution of AcChR

Functional reconstitution of the AcChR into membrane vesicles may be verified by the measurement of agonist-induced flux using radioactive tracer cations as described above. Hazelbauer and Changeux (1974) dissolved membrane fragments from T. Marmorata in sodium cholate, removed the detergent by dialysis, and then added crude Torpedo lipids to the AcChR solution. The resulting preparations showed an agonist-induced release of vesicle entrapped $^{22}\text{Na}^+$ in some,

but not all, cases. Reconstitution of agonist-initiated cation efflux using Triton X-100 solubilized AcChR that had been purified by affinity chromatography has also been reported (Michaelson and Raftery, 1974; Michaelson et al., 1976). In these experiments the magnitude of the flux response also varied from preparation to preparation. Furthermore, some attempts at reconstitution of the affinity purified receptor using similar procedures were unsuccessful (Karlin et al., 1975; McNamee et al., 1975). Recently, reconstitution of receptor function following dissolution of membrane vesicles in sodium cholate and removal of detergent by dialysis in the presence of exogenous phospholipid was reported (Epstein and Racker, 1978). However, the membranes used in this study contained polypeptides other than the four receptor subunits. Using membrane fragments depleted of non-AcChR peptides by alkali treatment (Neubig et al., 1979; Elliott et al., 1980), Wu and Raftery (1979) demonstrated that receptor function could be reproducibly reconstituted using preparations which contained only the four receptor peptides. A similar result has been reported by Changeux et al., (1979) using preparations which contained predominantly the 40,000 and small amounts of the 50,000 and 65,000 dalton polypeptides. However, one cannot conclude that only the 40,000 dalton polypeptide is needed for AcChR function since it has been shown (Moore et al., 1979a) that only a small number of active receptors are necessary to observe a maximal flux rate and Changeux et al. did not determine the concentration of active receptors in their reconstituted preparation.

9. Probes of the AcChR Ion Channel

The effects in vivo of several compounds whose primary site of interaction is distinct from the agonist binding site suggest that they may be useful probes for the membrane component or components related to ion translocation. One such compound is the alkaloid histrionicotoxin (HTX), first isolated by Daly et al., (1971). This compound has been postulated to block agonist-mediated depolarization by binding to and blocking the ion channel of the AcChR (Albuquerque et al., 1973a,b). It has also been suggested (Katz and Changeux, 1976; Burgermeister et al., 1977) that HTX blocks conduction by converting the receptor to a "desensitized" state. However, although HTX increases the apparent rate of desensitization in some tissues (Burgermeister et al., 1977) it has little or no effect on this process in others (Elliott and Raftery, 1977).

In vitro studies have shown that membrane fragments from Torpedo californica bind ^3H -labeled H_{12} -HTX in a specific manner with a K_d of 0.3-0.5 μM and a stoichiometry of 1 HTX site to 4 α -BuTx sites (Elliott and Raftery, 1977) in addition to a large non-specific binding component. HTX binding is lost on dissolution of membrane fragments using the detergents Triton X-100 and cholate. The loss is irreversible in the case of Triton but binding may be recovered in cholate solutions if the detergent is diluted to a concentration of 0.5% (Elliott and Raftery, 1979). Recently, it has been demonstrated (Elliott et al., 1979) that HTX binding requires only the four polypeptides characteristic of purified receptor.

Caeruleotoxin, an acidic polypeptide toxin from the venom of Bungarus caeruleus was considered as another candidate for a specific

probe of the "ionic conductance modulator" (Bon and Changeux, 1975). However, the post-synaptic action of this polypeptide has been attributed to its phospholipase activity (Moody and Raftery, 1978). Local anesthetics comprise a third class of potentially useful compounds for study of mechanisms related to ion channel function.

At the neuromuscular junction, local anesthetics alter the normal time course of end-plate currents induced by AcCh; whereas normal end-plate currents decay with a single exponential time course, the decay becomes multiphasic in the presence of procaine and other local anesthetics (Furukawa, 1957; Maeno, 1966, Gage and Armstrong, 1968; Steinbach, 1968a,b). Recent electrophysiological studies (Neher and Sakmann, 1976; Ruff, 1976; Steinbach, 1977) have led to proposals that this action might be explained by binding of a local anesthetic to the open channel (i.e., conducting) form of the AcChR, resulting in a decreased ionic conductance. These compounds, then, might be used as probes for the structure(s) involved in translocation of ions across the post-synaptic membrane and its relationship to the AcChR. Chapter Two describes studies on the interactions of various local anaesthetics with receptor-rich membrane fragments from Torpedo californica.

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CHAPTER 1: A Rapid and Reproducible Method for Preparation
of Acetylcholine Receptor Enriched Membrane Fragments from
Torpedo californica

Introduction

The electric organs of Torpedo californica have been used for the isolation of nicotinic acetylcholine receptors (AcChR) in both the membrane-bound state (Duguid and Raftery, 1973; Reed et al., 1975) and the detergent-solubilized state purified by affinity chromatography (Schmidt and Raftery, 1972). The purified receptor consists of four polypeptide subunits with apparent molecular weights of 40,000, 50,000, 60,000, and 65,000 as measured by SDS/polyacrylamide gel electrophoresis (Raftery et al., 1974; Weill et al., 1974; Vandlen et al., 1976). Studies of the solubilized AcChR provide limited information since both the ability of the receptor to undergo an agonist-induced increase in ligand affinity (Weber et al., 1975; Weiland et al., 1976; Lee et al., 1977) and the ion translocation function of the AcChR are lost on solubilization. Therefore biochemical studies on the ligand binding and ion translocation properties of the AcChR require a membrane-bound AcChR preparation.

A number of methods for the preparation of membrane fractions from various Torpedo species enriched in AcChR have been published (Cohen et al., 1972; Duguid and Raftery, 1973; Reed et al., 1975; Sobel et al., 1977). These studies used sucrose density gradients in swinging bucket, or zonal rotors to effect separation of AcChR containing membranes [$\rho \sim 1.18$ (Hartig and Raftery, 1979)] from less dense contaminating membranes. The centrifugation steps in these methods required considerable amounts of time [e.g., ~ 18 hr. for the large-scale zonal preparation of Reed et al. (1975)] thus increasing the

possibility of receptor degradation by calcium-activated proteases (H.-P. H. Moore and M. A. Raftery, unpublished) or perturbation of the membrane environment by calcium-activated phospholipases (W. Wu and M. A. Raftery, unpublished) present in the crude homogenates.

In this chapter a rapid and reproducible method for large-scale purification of AcChR-enriched membrane fragments is described. The method utilizes a sucrose step gradient which diffuses to a continuous, non-linear gradient during centrifugation in a reorienting rotor. The specific activity of the resulting membranes is comparable to that achieved by zonal centrifugation (Reed et al., 1975) while the yield per gram of tissue is increased three to four-fold.

Further purification of the membrane fragments by treatment with base at pH 11 as described by Steck and Yu (1973) for erythrocyte ghosts and extended to AcChR containing membranes (Neubig et al., 1979) resulted in preparations containing essentially only the polypeptides characteristic of the receptor purified by affinity chromatography. Evidence is provided that this treatment does not alter the ligand binding properties of the AcChR preparations.

Materials and Methods

(1) Materials

Torpedo californica was obtained locally. The electric organs were excised and either used immediately or frozen in liquid nitrogen and stored at -90°C until use. Lyophilized venom of Bungarus multicinctus, carb, DTT and IAcNH_2 were from Sigma Chemical Company. $\text{Na } ^{125}\text{I}$ was from New England Nuclear and Whatman DE-81 DEAE-cellulose filter discs were purchased from VWR Scientific Co. $[^{125}\text{I}] \alpha\text{-BuTx}$ was prepared as described by Blanchard et al. (1979) $[^3\text{H}]$ -carb was the gift of Dr. Y. Chao. All other materials were of the highest purity commercially available.

(2) DEAE Disc Assay

The DEAE disc assay of Schmidt and Raftery (1973) was used to determine the concentration of (solubilized or membrane-bound) AcChR and to follow the kinetics of toxin binding. For the determination of toxin sites, 100 μL of solutions containing $[^{125}\text{I}] \alpha\text{-BuTx}$ (concentration $\approx 1 \times 10^{-7} \text{ M}$) and varying amounts of receptor were applied to DE-81 DEAE discs after a 10-30 min incubation period at room temperature. The discs were then washed with three changes, over 30 min, of washing buffer (10 mM sodium phosphate, pH 7.5, 0.1% Triton X-100, and 50 mM sodium chloride). The discs were counted in a Beckman 4000 γ counter or in a Packard Tri-Carb Model 3375 scintillation spectrometer. When the kinetics of $[^{125}\text{I}] \alpha\text{-BuTx}$ binding to receptor (e.g., in the presence of ligand) were measured, the AcChR was incubated for 20-30 min with the desired concentration

of the ligand. [^{125}I]- α -BuTx (in excess over receptor) was then added to start the reaction. Aliquots (100 μL) were then taken at given time intervals and pipetted onto DEAE discs which were subsequently transferred into washing buffer and treated as described above. The counts per minute at time t , C_t , are proportional to the amount of receptor-[^{125}I]- α -BuTx complex formed plus a constant additive background, C_b , due to nonspecific binding of toxin (C_b was of the order of 0.5% of the total counts present in the sample). Since the difference $C_\infty - C_t$ (C_∞ = counts per minute measured after completion of the reaction) was considered, the (constant) background canceled.

This method gives a valid measure of the rate of toxin binding only if the reaction is greatly slowed by addition of sample to a DEAE disc. (One might expect that the reaction will be quenched by competition of the high concentration of diethylaminoethyl groups on the disc with toxin for receptor.) That the reaction was indeed quenched upon contact with the DEAE disc was shown as follows. The apparent rate of α -BuTx binding to membrane-bound AcChR was first determined by the DEAE filter disc assay described above. Another receptor-toxin reaction using the same concentrations was then started, and for each time point an aliquot of the reaction mixture was pipetted into a large excess of unlabeled α -BuTx. This resulted in an essentially instantaneous conversion of all free receptor to unlabeled receptor-toxin complex. Because the receptor-toxin reaction is irreversible, the fraction of total receptor-toxin complex due to [^{125}I]- α -BuTx was just the amount of [^{125}I]- α -BuTx-receptor complex formed at the time the reaction was quenched. This concentration was measured by the

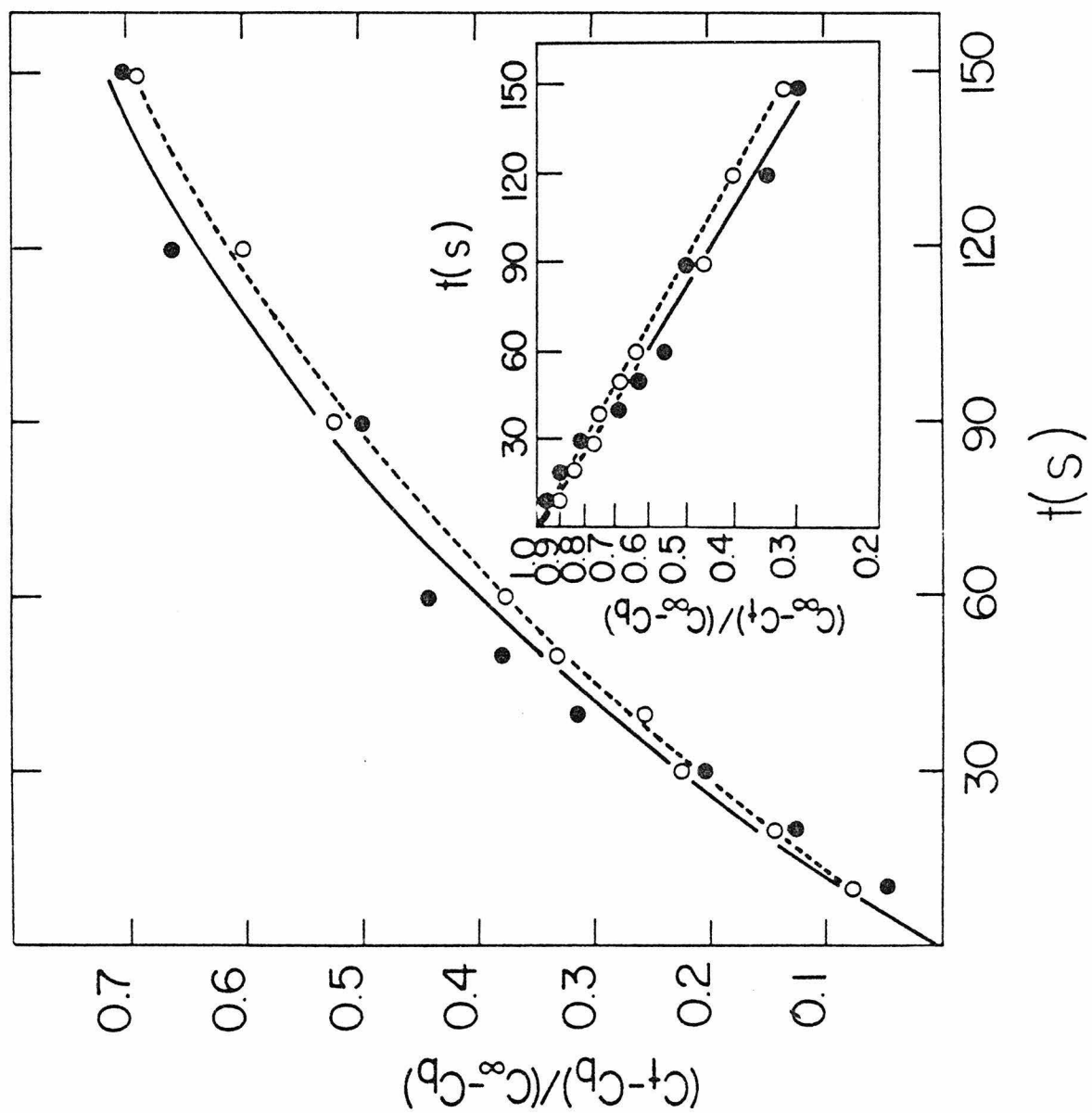
method of Schmidt and Raftery (1973). Figure 1 shows that the apparent rates of [125 I]- α -BuTx binding obtained in the two experiments agreed within 8%, indicating that the DEAE filter assay gives an accurate measure of the toxin binding rate.

(3) Assays

The rate at which the AcChR underwent the low to high affinity transition for agonists was determined from the time-dependent increase in inhibition of [125 I]- α -BuTx binding caused by incubation with a fixed concentration of carb (Lee et al., 1977; Quast et al., 1978). Protein concentrations were determined by the method of Lowry et al., (1951).

Equilibrium binding of [3 H]-carb was measured by centrifugation assay as follows: Membrane fragments 0.25 μ M in α -BuTx sites were incubated with varying concentrations of [3 H]-carb for 30 min at room temperature. Duplicate 0.25 ml samples (1.5 ml total volume) were taken before and after centrifugation for 15 min in an Eppendorf 3200 centrifuge. Radioactivity was determined in a Packard 3375 scintillation counter using 8 ml Aquasol-2 (New England Nuclear) per sample. Non-specific binding was determined in duplicate tubes to which excess unlabeled carb was added. In most cases the non-specific component was less than 2% of the total bound and could be ignored. Bound and free concentrations of the ligand were calculated and the data plotted according to Scatchard (1949).

FIGURE 1: Demonstration of quenching [^{125}I]- α -BuTx binding to AcChR by the DEAE disc assay. The rate of toxin binding was measured by the DEAE disc assay as described under Methods. The data are presented in a normalized form ($\bullet$) where C_∞ represents the counts per minute bound to a disc at equilibrium and C_b represents a constant, additive background. The reaction mixture was $0.5\ \mu\text{M}$ in [^{125}I]- α -BuTx and $55\ \text{nM}$ in α -toxin binding sites. Another reaction, at the same concentration, was started. For each time point, a $50\text{-}\mu\text{L}$ aliquot was pipetted into $75\ \mu\text{L}$ of $50\ \mu\text{M}$ unlabeled α -BuTx with rapid mixing. After all the points had been taken, $100\ \mu\text{L}$ from each point was pipetted onto a DEAE disc and treated as described (see Methods). C_∞ was determined in the same way, while C_b was determined from the intercept ($C_\infty - C_b$) of the semilogarithmic plot of the data. The data ($\circ$) are shown as a normalized plot to allow direct comparison with the data determined from the DEAE disc assay ($\bullet$). The lines were drawn with the rate constants determined by linear least-squares analysis of the semilogarithmic plot of the data. (Insert) Normalized, semilogarithmic plots of the data. The second-order rate constants as determined by linear least-squares analysis of the data were $(1.64 \pm 0.08) \times 10^4\ \text{M}^{-1}\ \text{s}^{-1}$ for the disc assay ($\bullet$) and $(1.53 \pm 0.04) \times 10^4\ \text{M}^{-1}\ \text{s}^{-1}$ in the case where the reaction was stopped by an excess of unlabeled toxin ($\circ$).



(4) Preparation of Crude Membrane Fragments

In a typical preparation 500 g of frozen electric organ was broken into small pieces and an equal volume of cold (4° C) homogenization buffer was added (0.4 M NaCl, 5mM EDTA, 50 mM sodium phosphate, pH 7.8, 0.02% NaN₃). Crystalline phenylmethanesulphonyl fluoride and IAcNH₂ (if desired) were added to give final concentrations in the homogenate of 10 mM and 5 mM, respectively. The organ was ground for 2 min at high speed in a Waring blender under a stream of argon. The homogenate was reground in ~ 300 ml portions at 30,000 rpm in a Virtis Type 60 homogenizer for four periods of 30 sec each. Large particles of connective tissue were removed by centrifugation at 5000 rpm for 10 min in a Sorvall GSA rotor. The supernatant was poured through two layers of cheesecloth and centrifuged for 1 hr at 16,000 rpm in a Beckman Type 35 rotor at 2° C. The pellet was re-suspended in 10 mM sodium phosphate (pH 7.4)/1 mM EDTA/0.02% (w/v) NaN₃ using two 30 sec bursts of 30,000 rpm in the Virtis homogenizer. Sucrose and NaCl were added to the resulting membrane suspension to give final concentrations of 30% (w/w) and 2.3% (w/w) (400 mM), respectively.

(5) Fractionation of Membrane Fragments by Sucrose Gradient Centrifugation

Discontinuous sucrose gradients consisting of 5 ml of 50% (w/w), 5 ml of 39% (w/w), and 12 ml of 35% (w/w) sucrose in 0.4 M NaCl/1 mM EDTA/10 mM sodium phosphate (pH 7.4)/0.02% NaN₃ were layered in each of eight centrifuge tubes. 10 ml of crude membrane

fragments were added to each tube and the tubes were filled to a total volume of ~ 34 ml using an overlay of the above buffer without sucrose. Centrifugation was for 1 hr at 45,000 rpm in a Beckman VTi-50 rotor using a Beckman L-5B with slow acceleration accessory. A Sorvall TV-850 rotor gave an identical separation. Following centrifugation the tubes were found to contain three light scattering bands: heavy bands near the top and middle of the gradients with a lighter band near the bottom. The bands were collected by aspiration, diluted with sucrose free buffer and pelleted by centrifugation at 30,000 rpm for 1 hr in a Type 35 rotor. The pellets were resuspended in calcium free Ringers buffer and assayed for α -BuTx binding activity and protein.

(6) Treatment of Membrane Fragments with Alkali

Removal of non-receptor proteins from Torpedo membrane fragments was essentially as described by Steck and Yu (1973) for erythrocyte ghosts. In initial experiments, membranes from the middle band of the sucrose gradients were pelleted and resuspended in ice cold distilled water at a concentration of ~ 2 mg/ml and adjusted to pH 11 with NaOH. The membranes were immediately pelleted (100,000 xg) and resuspended in Ca^{++} -free Torpedo Ringers (250 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , 20 mM HEPES, pH 7.4, 0.02% NaN_3). For most experiments, however, base treatment was as described by Wu and Raftery (1979). Two additional features of this method ensured maximum specific activity of the treated membranes. These features were: i) The membranes were stirred for 1 hr at 4°C after adjustment to pH 11; and ii) The membranes were pelleted by centrifugation for

45 min at 18,000 rpm in a Sorvall SS-34 rotor. Under these conditions the pellet consisted of a "hard" dense pellet containing AcChR membrane fragments of high specific activity and a "soft" pellet which contained primarily non-receptor (43 and 90 K dalton) polypeptides (Wu and Raftery, 1979). The supernatant and "soft" pellet were decanted leaving the high specific activity membranes which were resuspended in Ca^{++} free Ringers buffer.

(7) Polyacrylamide gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using the buffer system of Laemmli (1970). The separating gel was 8.75% acrylamide, 0.23% bis-acrylamide and the stacking gel 3% and 0.08% in acrylamide and bis-acrylamide, respectively. The gels were stained 0.05% Coomassie Brilliant Blue, 25% MeOH, 10% HOAc and destained in the same solution lacking the dye. Gels were scanned at 550 nm using a Gilford 240 spectrophotometer with a linear transport unit.

Results

(1) Homogenization of Torpedo Electric organ

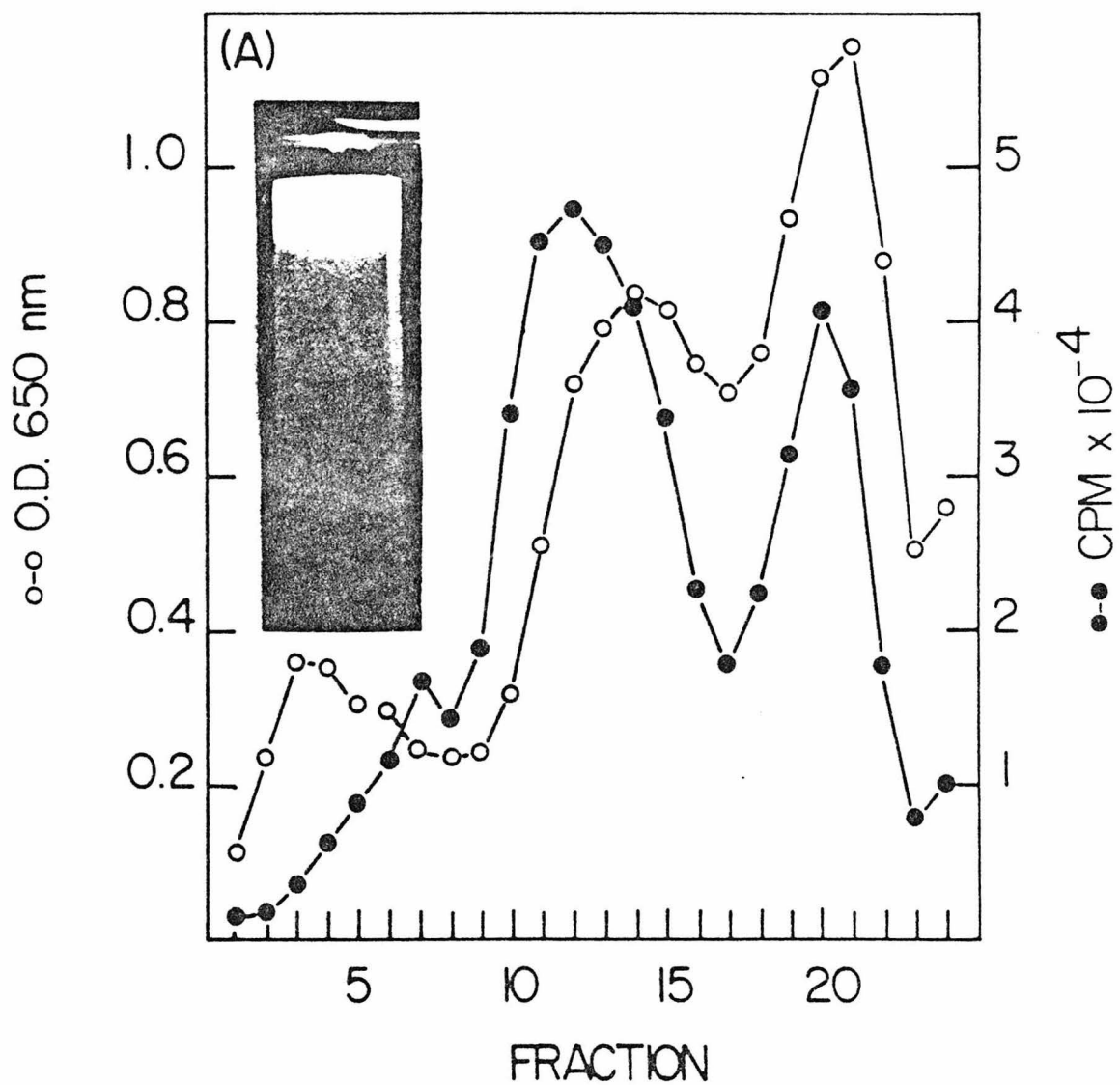
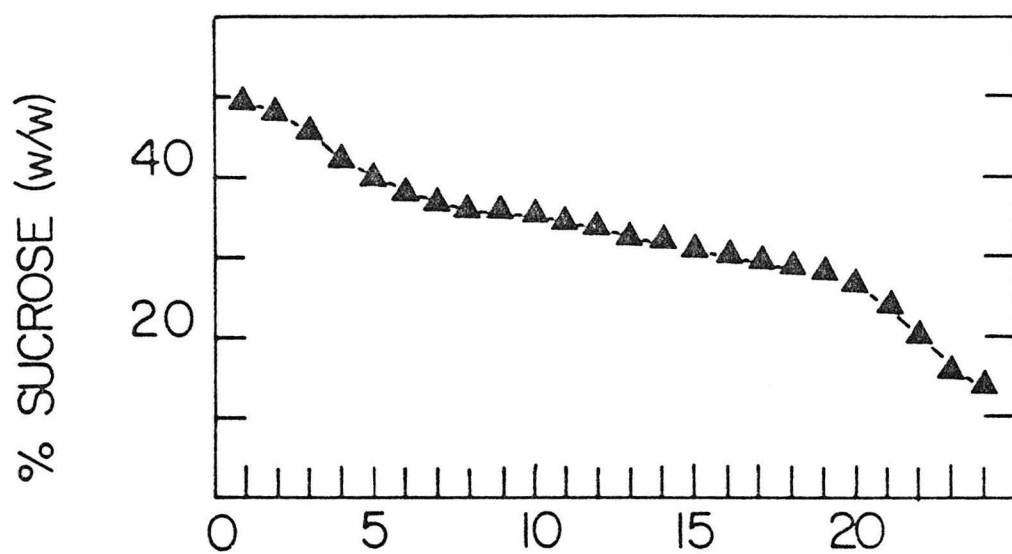
The homogenization conditions described in Methods were chosen to give maximal concentration of [^{125}I] α -BuTx binding activity in the supernatant of the 5000 rpm GSA centrifugation. Homogenization with a 1:1 ratio of tissue:buffer (compared to a ratio of 2:1) gave approximately a two-fold increase in total [^{125}I] α -BuTx binding activity released per gram of tissue. The re-grind in the Virtis apparatus was necessary to eliminate large pieces of intact electric tissue which were observed following the initial homogenization.

Homogenization of the frozen organ (as compared to thawed) also resulted in an increased yield of [^{125}I] α -BuTx binding sites ($\sim$ two-fold increase in the total number of sites present in the middle band of the sucrose gradient) with the specific activity remaining constant. This increase could be due to preservation of binding sites that were formerly lost during the long thawing process.

(2) Reorienting Gradient Centrifugation

A typical separation of Torpedo membranes on the reorienting sucrose gradient is shown in Fig. 2. The band centered around fraction 12 contained the majority of the α -BuTx binding activity while the major protein band was found near the top of the gradient with a peak near fraction 21. The membranes of the middle band, having the highest specific activity, were collected and diluted to give a sucrose concentration below the membrane equilibrium density and then pelleted.

FIGURE 2: Separation of *T. californica* membrane fragment populations by sucrose gradient centrifugation in a reorienting rotor:
The lower panel shows profiles of [^{125}I]- α -BuTx binding (●) and protein (○) from a single tube of a typical vertical rotor preparation. Sucrose concentrations (▲) measured by refractometry are indicated in the upper panel. Insert: Photograph of the tube following centrifugation showing the presence of three light-scattering bands corresponding to the three protein bands of the profile.



The pellet was resuspended in Ca^{++} -free Torpedo Ringers and used for further studies of the AcChR.

Although the specific activity of the membranes varied somewhat from preparation to preparation the ratio of the specific activities of the three light-scattering bands was highly reproducible at approximately 1:4:1 top:middle:bottom (Table 1). Furthermore, when membrane fragments from the middle band were run on a 30%-50% (w/v) sucrose gradient in a zonal rotor as previously described (Reed et al., 1975) no significant increase in specific activity was observed.

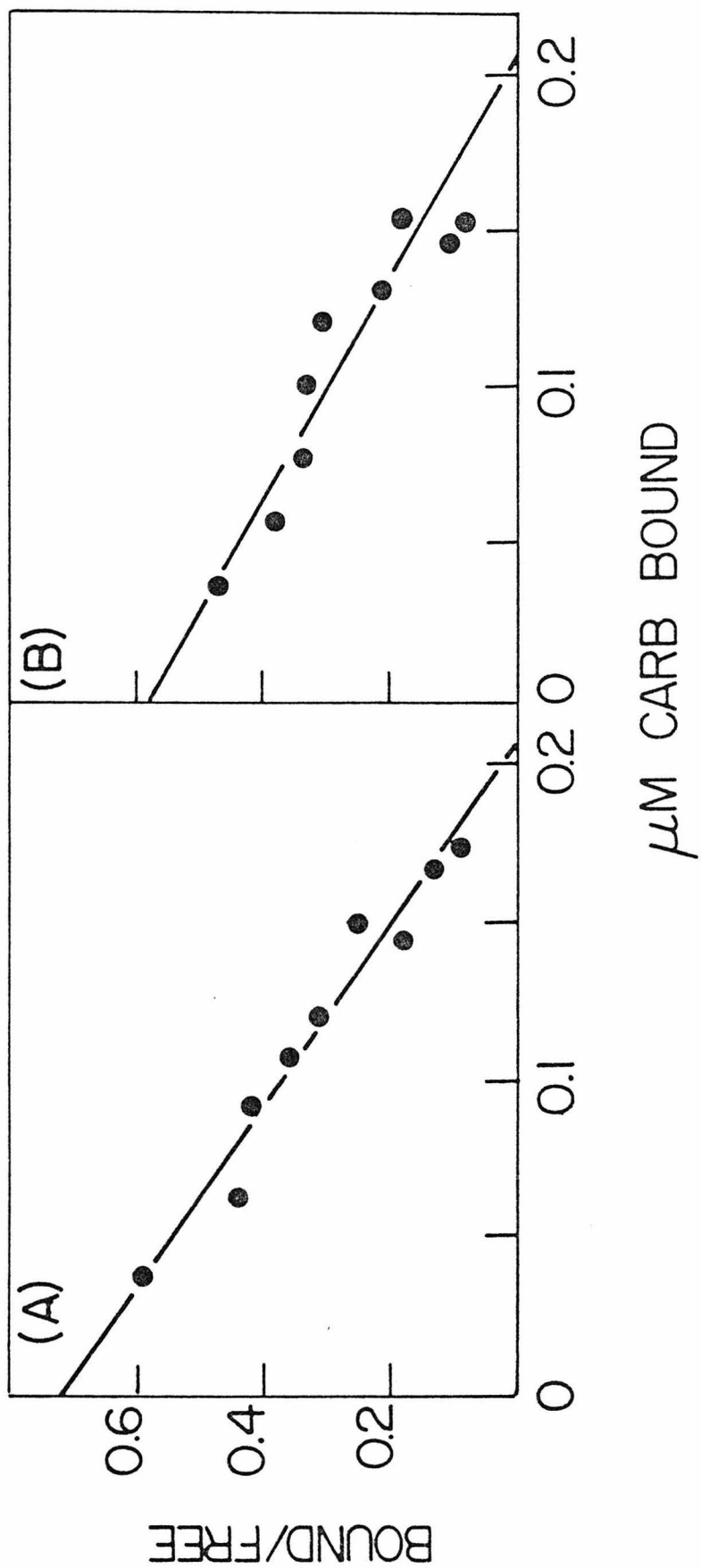
(3) Effects of Iodoacetamide

Inclusion of 5 mM IAcNH₂ during the homogenization of the electric organ resulted in a consistent two-fold increase in the specific activity of the membrane fragments (i.e., middle band) obtained after reorienting sucrose gradient centrifugation. Iodoacetamide treatment did not perturb the ligand binding properties of the AcChR. The rate of [¹²⁵I]α-BuTx binding was $\sim 2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for iodoacetamide treated membranes in excellent agreement with previous studies (Quast et al., 1978; Blanchard et al., 1979). Figure 3 shows Scatchard plots of [³H]-carb binding to membrane fragments prepared in the presence and absence of IAcNH₂ in the grinding buffer. It is apparent that neither the dissociation constant ($0.29 \pm 0.02 \text{ } \mu\text{M}$ and $0.35 \pm 0.05 \text{ } \mu\text{M}$, respectively) nor the number of binding sites ($0.21 \pm 0.02 \text{ } \mu\text{M}$ and $0.20 \pm 0.04 \text{ } \mu\text{M}$) were significantly affected by treatment with iodoacetamide.

TABLE I: Distribution of [^{125}I] α -BuTx Binding and Protein After Reorienting Sucrose Gradient Centrifugation of Torpedo Membranes

FRACTION	mg PROTEIN	nmoles [^{125}I] α -BuTx BINDING SITES	SPECIFIC ACTIVITY ($\frac{\text{nmoles } [^{125}\text{I}] \alpha\text{-BuTx sites}}{\text{mg PROTEIN}}$)	% OF STARTING MATERIAL RECOVERED	
				PROTEIN	TOXIN BINDING
STARTING MATERIAL	434	291	0.67	43.5	20.6
TOP	189	60	0.32		
MIDDLE	80	108	1.35	18.4	37.1
BOTTOM	74	36	0.49	17.1	12.4

FIGURE 3: [³H]-carb binding to membrane fragments prepared in the absence and presence of IAcNH₂: Membrane fragments were 0.25 μM in [¹²⁵I]-α-BuTx binding sites. The data were plotted according to Scatchard (1949) and the values obtained by linear least squares (solid lines) were: (A) No IAcNH₂, Kd = 0.29 ± 0.02 μM; Ro = 0.21 ± 0.02 μM, and (B) IAcNH₂ treated, Kd = 0.35 ± 0.05 μM; Ro = 0.20 ± 0.04 μM.



(4) Effects of Modification of the Gradient on Separation of Membrane Fragment Populations

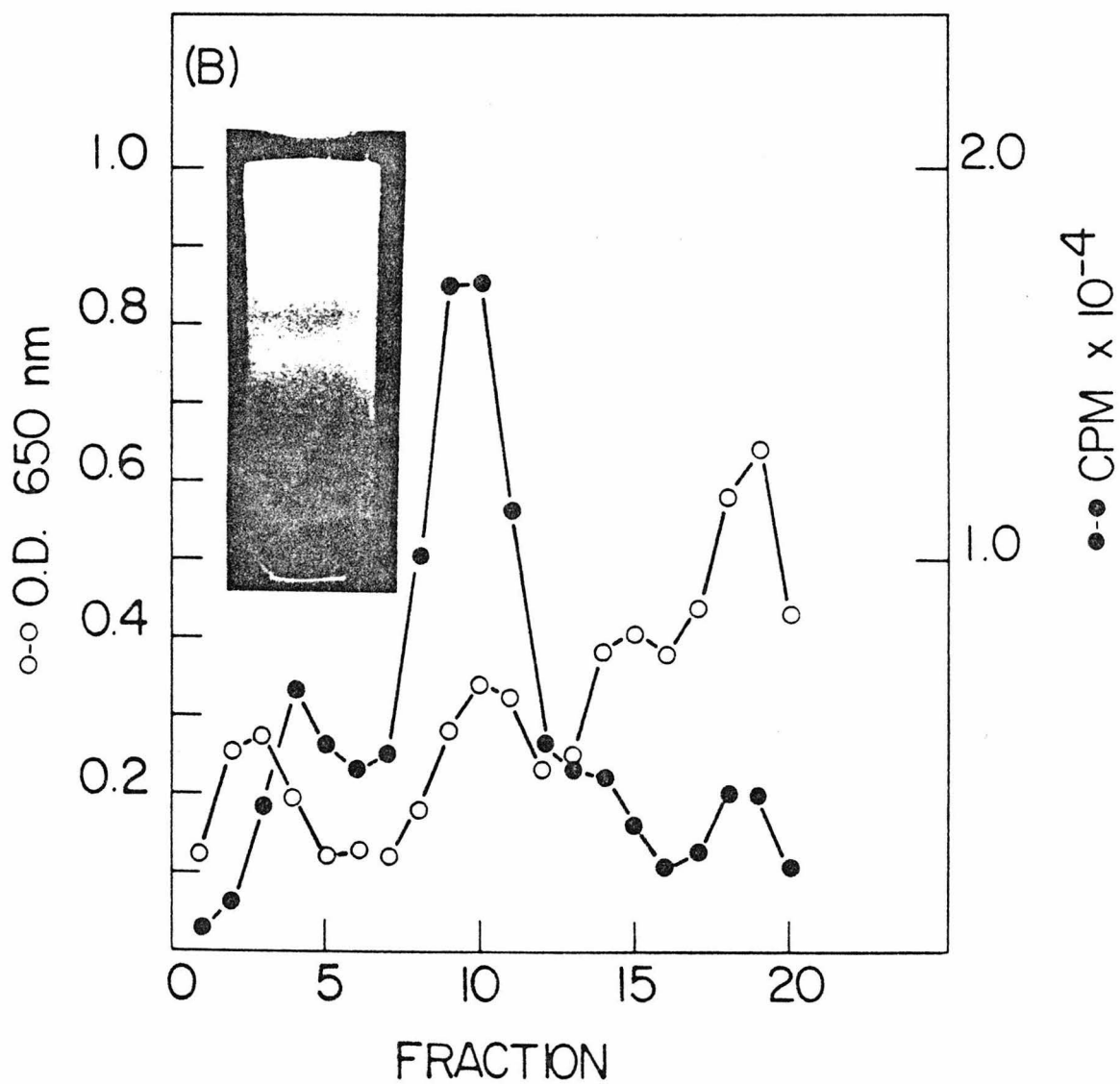
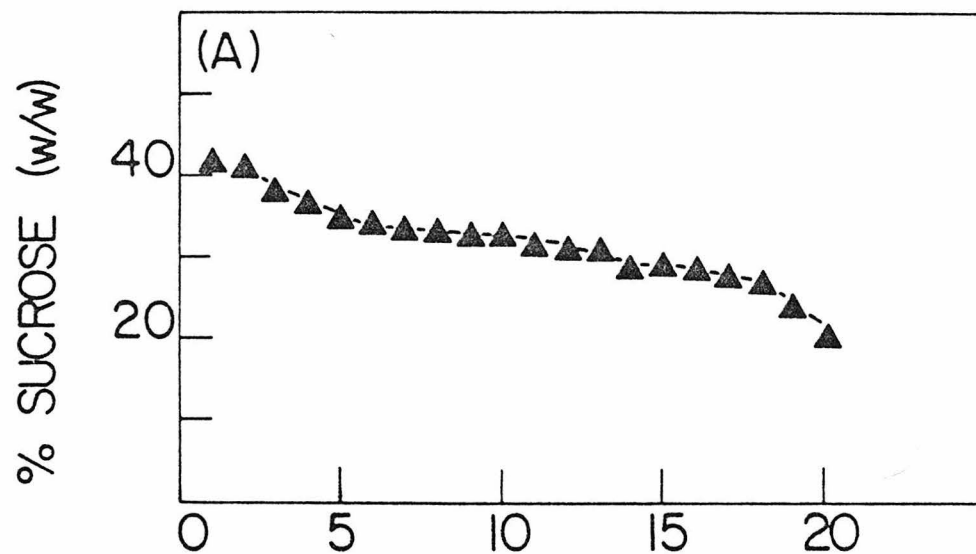
The separation achieved by reorienting sucrose density gradient centrifugation was only slightly affected by small changes in the relative volumes of the 35% (w/w) and the sample (30% (w/w)) layers. For instance, when the volume of the 35% (w/w) layer was decreased from 12 ml to 10 ml, with a corresponding increase in the volume of the sample band, three light scattering bands were still observed. The relative specific activities of these bands were unchanged, but their positions in the tube relative to each other were slightly shifted. If, however, the 39% (w/w) layer was omitted and the volume of the 30% (w/w) (sample) layer increased by a corresponding amount, a slightly different pattern was observed after centrifugation. A new light-scattering band was now seen between the top and middle bands (Fig. 4).

The peaks for protein and toxin binding were observed to be slightly skewed in the original gradient (Fig. 2), but this was not the case for the shallower gradient. In the shallow gradient the middle band was resolved into two fractions with the denser fraction having the higher specific activity. This modified gradient was therefore routinely used for membrane fragment purification.

(5) Further Purification of Reorienting Membrane Fragments by Treatment with Base

As shown by polyacrylamide gel electrophoresis, extraction of peripheral membrane proteins from reorienting membrane fragments

FIGURE 4: Increased resolution of *T. californica* membrane fragments on sucrose density gradients: After centrifugation on the modified gradient [39% (w/w) sucrose replaced by 30% (w/w)] as described in the text, a single tube was fractionated and the individual fractions were assayed for: (a) sucrose concentration by refractometry and (b) [125 I]- α -BuTx binding (●) and protein (○). The inset shows the separation of the membrane fragments after centrifugation. The four light-scattering bands correspond to the four protein peaks observed in the profile.



by treatment at pH 11 resulted in a preparation which contained essentially only the four polypeptides characteristic of solubilized, purified receptor (Fig. 5). This treatment did not, however, affect the ligand binding properties of the AcChR.

Base treated membrane fragments bound [^{125}I] α -BuTx with a rate nearly identical to that of the untreated membranes ($2.6 \pm 0.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ versus $2.9 \pm 0.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$; Fig. 6). Both the dissociation constant and amount of carb bound at equilibrium (Fig. 7) for the base treated membranes were consistent with typical values for the untreated membranes (see, e.g., Fig. 2). The rate of the carb-induced increase in receptor affinity for agonists as measured by the time-dependent increase of [^{125}I] α -BuTx binding was also unchanged (Fig. 8).

FIGURE 5: Densitometer scans of Coomassie Brilliant Blue stained sodium dodecyl sulfate/polyacrylamide gels of membranes before (a) and after (b) treatment of pH 11. The 40,000, 50,000, 60,000, 65,000 and 90,000 dalton polypeptides are indicated in (a).

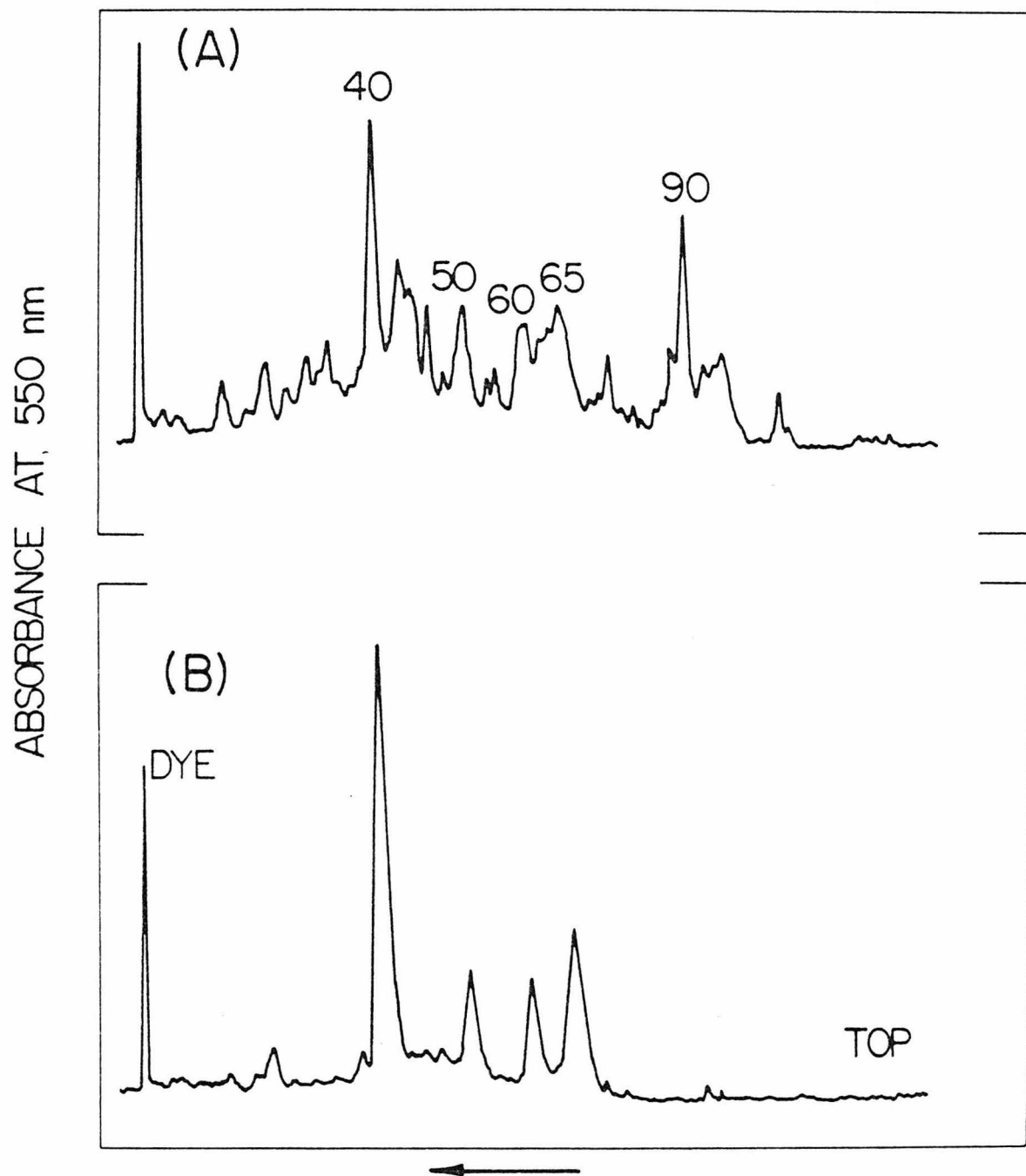


FIGURE 6: The rate of [125 I]- α -BuTx binding to membrane fragments with toxin in excess. The concentrations were 0.063 μ M [125 I]- α -BuTx and 0.84 μ M in toxin binding sites. The rates were calculated from the slope of linear least-squares fits of $\ln (C_{\infty} - C_t)$ versus time where C_{∞} is the cpm bound at equilibrium and C_t is the cpm bound at time t as measured by the disc assay of Schmidt and Raftery (1973).
 (●—●) untreated membranes $K = 2.6 \pm 0.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and (o--o) membranes treated with base at pH 11, $k = 2.9 \pm 0.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$.

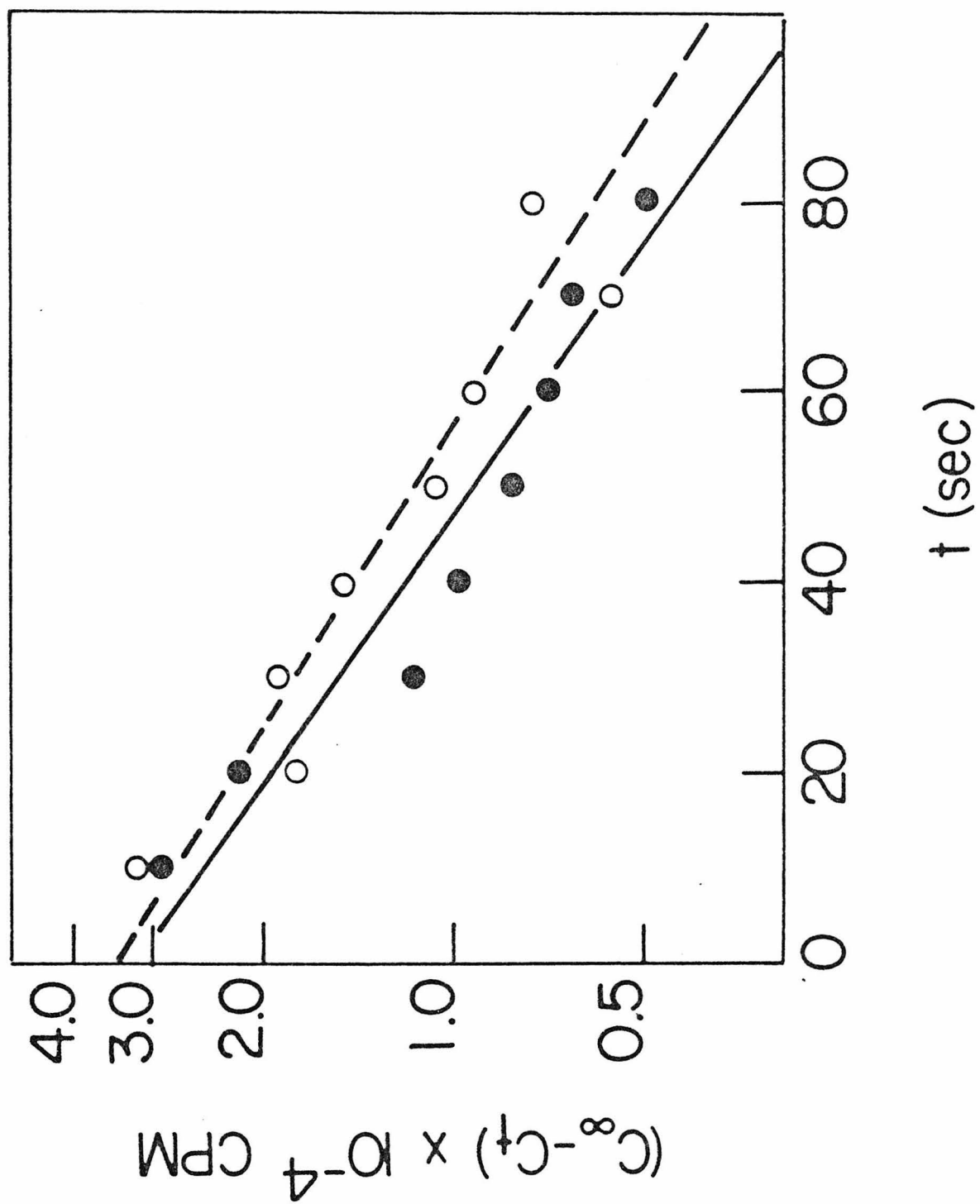


FIGURE 7: [³H]-carb binding to membranes treated at pH 11.
The concentration of [¹²⁵I]- α -BuTx sites was 0.25 μ M. Linear least-squares of the data plotted according to Scatchard (1949) gave a dissociation constant of 0.16 ± 0.02 μ M and a value of 0.27 ± 0.04 μ M for the number of carb binding sites.

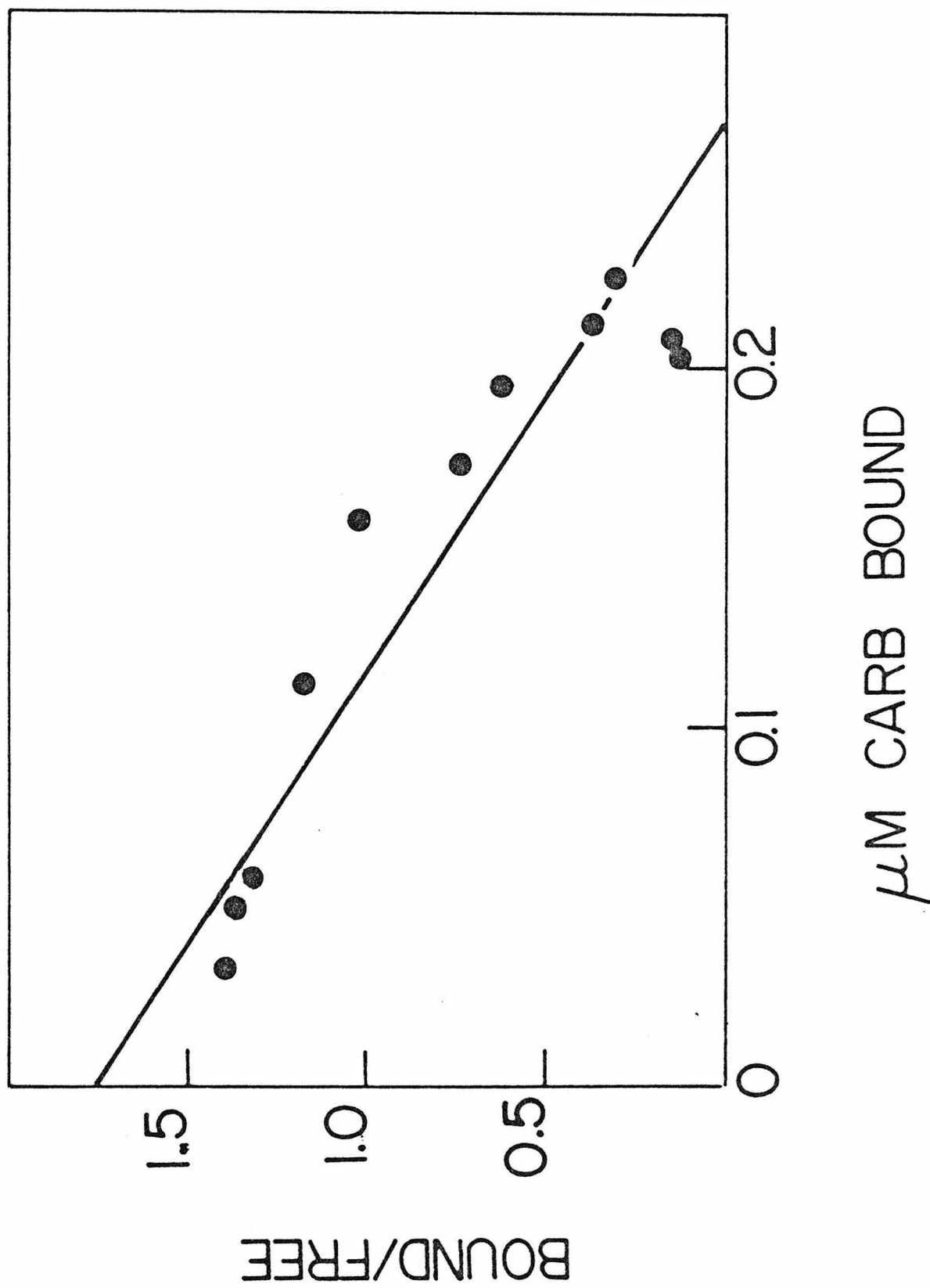
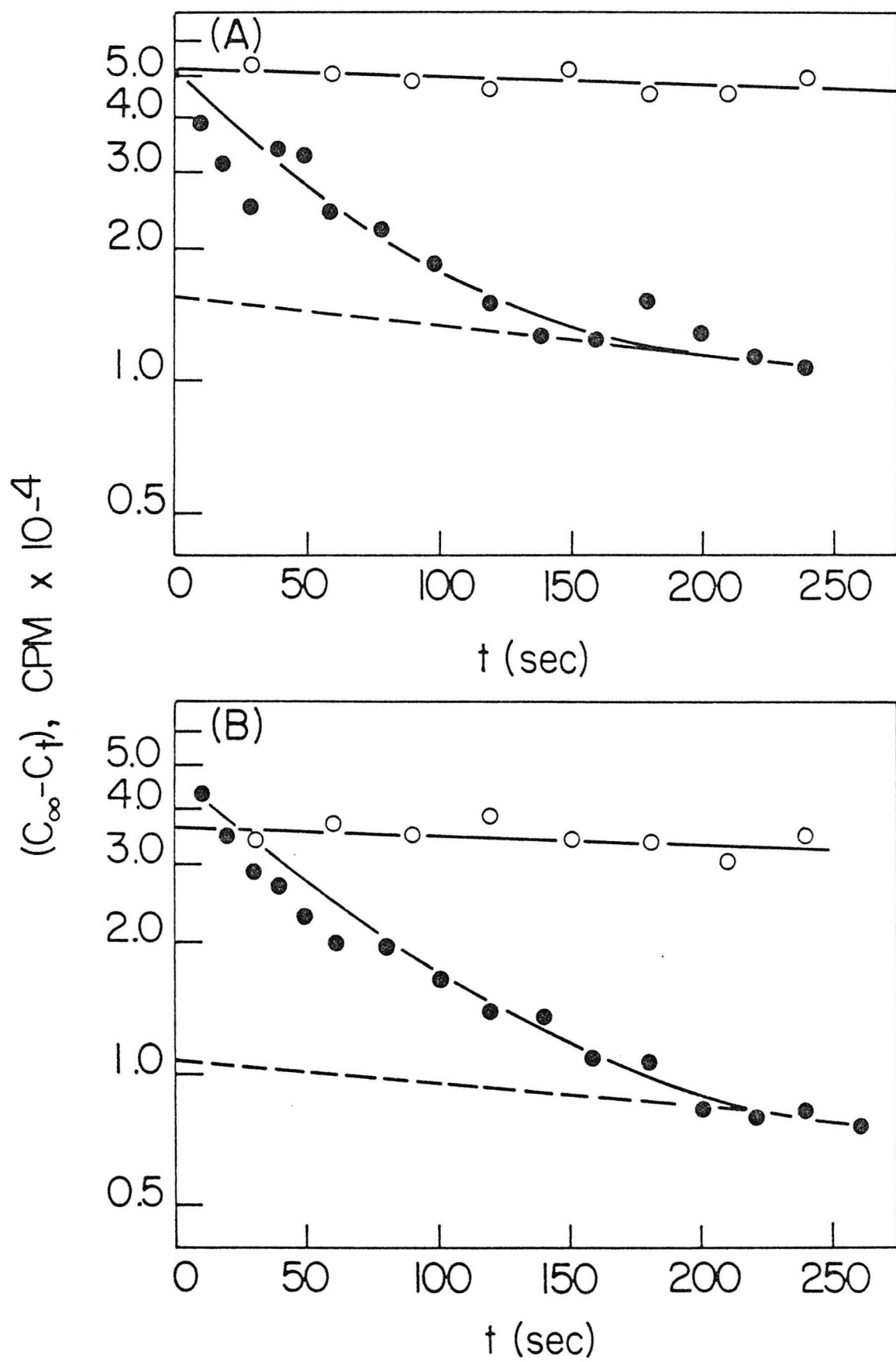


FIGURE 8: Rate of transition of AcChR to a high affinity form.

Receptor was in excess over $[^{125}\text{I}]\text{-}\alpha\text{-BuTx}$. The concentrations used were: AcChR = $0.84\ \mu\text{M}$; $[^{125}\text{I}]\text{-}\alpha\text{-BuTx}$ = $0.063\ \mu\text{M}$ and Carb = $5\ \mu\text{M}$. (○) Rate of toxin binding to receptor completely in the high affinity form as a result of a 20-minute incubation of receptor with carb before addition of $[^{125}\text{I}]\text{-}\alpha\text{-BuTx}$. (●) Time course of $[^{125}\text{I}]\text{-}\alpha\text{-BuTx}$ binding for simultaneous addition of carb and toxin to AcChR. The fast initial decrease reflects the transition to the high affinity form while the linear portion at the end of the curve shows toxin binding to receptor completely in the high-affinity form. A semilog plot of the difference between the actual data points and the limiting slope at long times (---) gave a direct measure for the rate of transition (Lee et al., 1977; Quast et al., 1978). The values obtained by linear least-squares gave half-times for the transition of (A) untreated membranes = $74 \pm 22\ \text{sec}$ and (B) base treated membranes = $73 \pm 5\ \text{sec}$.



Discussion:

The use of reorienting sucrose gradient centrifugation as described above permits rapid purification of a membrane fraction from homogenates of Torpedo electric organ enriched in nicotinic acetylcholine receptor. The short time required for preparation of the membrane fragments coupled with the use of EDTA to inhibit the activity of calcium-activated proteases and phospholipases present in Torpedo electric tissue homogenates (H.-P. H. Moore, W. C.-S. Wu and M. A. Raftery, unpublished observations) results in a highly reproducible preparation suitable for further studies on the AcChR. In addition treatment of membranes at pH 11 gives a functional preparation with specific activities approaching those obtained for detergent-solubilized receptor purified by affinity chromatography.

Improved techniques of homogenization, specifically an increase in the ratio of buffer: electric organ and homogenization of the frozen organ, results in a greatly increased yield of total α -BuTx binding activity. Approximately a four-fold increase in the number of [125 I] α -BuTx sites obtained per gram of electric organ is achieved as compared to earlier methods (Duguid and Raftery, 1973; Reed et al., 1975). A high yield of AcChR from a single preparation is especially useful for studies which require a large amount of material such as ligand binding studies using stopped-flow methods or studies of agonist mediated ion flux.

Iodoacetamide treatment resulted in an increased specific activity of the membrane fragments, presumably by alkalating free

sulphydryl groups, thus preventing the formation of nonspecific disulfide bonds between the polypeptides in AcChR-containing membranes and other, non-related proteins. An additional result of iodoacetamide treatment was the stabilization of the receptor in its 13S dimeric form (Chang and Bock, 1977; Hamilton et al., 1977; Witzemann and Raftery, 1978). This treatment did not affect either [^{125}I] α -BuTx or carb binding properties. In addition, the rate of the transition of receptor from a state of low to high affinity, HTX binding (Elliott et al., 1980) and agonist-induced flux of $^{22}\text{Na}^+$ (Miller et al., 1979) were also unaffected.

Treatment of membranes with alkali at pH 11 resulted in a membrane preparation containing essentially only the 40,000, 50,000, 60,000, and 65,000 dalton polypeptide species characteristic of solubilized, purified AcChR. This highly purified membrane fraction exhibits normal α -BuTx and carb binding. In addition, this preparation has been used to demonstrate that only those four polypeptides characteristic of the purified AcChR are necessary for binding of HTX (Elliott et al., 1979; Elliott et al., 1980) and for agonist-induced cation translocation (Moore et al., 1979). Neubig et al. (1979) have also reported that a similar preparation which lacked a 43,000 dalton component present in the untreated membranes specifically bound an HTX competitive local anesthetic analog. However, the initial membrane preparation of Neubig et al. differed from our preparations in that it did not contain a 60,000 dalton polypeptide. These results are important since a 43,000 M.W. component in T. marmorata membranes has been implicated as the HTX binding site (Sobel et al., 1978) and HTX is believed to function by blocking the ion channel (Albuquerque et al., 1973a, b).

Also, a 43,000 M.W. component in T. californica membranes is labeled by a photolabile local anesthetic analog. However, HTX, α -BuTx, and carb did not protect against labeling so that this component may be unrelated to the AcChR (see Chapter 3).

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CHAPTER 2: Interactions of Local Anesthetics with Torpedo
californica Acetylcholine Receptor

Introduction

The ligand binding properties of the AcChR have been studied in vitro by using membrane fragment preparations enriched in this molecule obtained from several species of marine electric ray (Cohen et al., 1972; Duguid and Raftery, 1973; Reed et al., 1975). However, the ion translocation function of the AcChR has not been as extensively characterized in vitro. In vivo, local anesthetics block the normal response of the AcChR to the binding of agonists, and electrophysiological studies of the actions of local anesthetics on the AcChR (Ruff, 1976; Neher and Sakmann, 1976; Steinbach, 1977) have led to proposals suggesting that these compounds act by binding to the open-channel form of the AcChR, thus decreasing the ionic conductance. In addition, local anesthetics increase the rate of pharmacological desensitization (Magazanik and Vyskocil, 1973; Magazanik, 1976), thus facilitating the conversion of the receptor to a nonconducting state. Upon prolonged exposure to agonists, membrane-bound AcChR in vitro is converted to a state of high affinity for agonists (Weber et al., 1975; Weiland et al., 1976, Lee et al., 1977, Quast et al., 1978) and it has been suggested that this process is an in vitro correlate of desensitization in vivo. Therefore, studies of the action of local anesthetics on AcChR function in vitro may provide information on the process of ion translocation.

The experiments reported here detail the effects of local anesthetics on AcChR-enriched membrane fragments from Torpedo californica with respect to (a) inhibition of the rate of [125 I]- α -BuTx-AcChR complex formation, (b) changes in the rate of the agonist-induced increase in receptor affinity, as measured by the inhibition of

[¹²⁵I]- α -BuTx binding in the presence of carb, and (c) local anesthetic induced conformational changes in AcChR membranes as monitored using the fluorescent probe ethidium bromide. Equilibrium studies showed that most local anesthetics affected the fluorescence of AcChR-Eth complex in a complicated manner: low concentrations of local anesthetic increased the fluorescence of membrane bound Eth while higher concentrations quenched this fluorescence (Schimerlik and Raftery, 1976; Schimerlik et al., 1979a). The local anesthetic tetracaine, however, caused only a decrease in fluorescence intensity. Because local anesthetics affected the fluorescence of Eth specifically associated with the AcChR and since these compounds did not displace specifically bound [³H] Eth, it seemed to be a suitable indicator for the study of the kinetics of local anesthetic interactions with the membrane-bound AcChR.

Materials and Methods

(1) Preparation of Membrane Fragments

AcChR-enriched membrane fragments were prepared as previously described (Duguid and Raftery, 1973; Reed *et al.*, 1975) or by the reorienting sucrose gradient method of Chapter 1. The determination of [125 I]- α -BuTx sites, protein concentration, and the rate of α -toxin binding by the DEAE-disc assay were as described in Chapter 1.

(2) Determination of the Rate of the in vitro Affinity Change

Membrane fragment preparations were routinely tested for their ability to undergo the agonist-induced transition from a state of low to high affinity for cholinergic ligands by comparing the rate of [125 I]- α -BuTx binding in the presence of 1 μ M carb with and without 15 min of incubation (Lee *et al.*, 1977). Preparations in which a significant amount of the receptor was initially in a state of high affinity were discarded. For measurement of the rate of the receptor transition from the low- to high-affinity state for agonists, membranes were incubated with the desired anesthetic for 15-20 min at room temperature followed by addition of carb (1 μ M final concentration). After a given time, [125 I]- α -BuTx was added and the rate of toxin-receptor complex formation, k_{app} , was determined as described above. The variation of k_{app} vs. time of incubation was fit to an exponential decay of the form

$$k_{app}(t) = (k_0 - k_{\infty})e^{-k't} + k_{\infty} \quad (1)$$

where k_0 was the rate of toxin binding in the presence of 1 μ M carb

without preincubation, k_{∞} was the rate of toxin binding in the presence of 1 μ M carb when all of the receptor was in the high-affinity state (for the purposes of these experiments, this condition was fulfilled after 15 min of preincubation), and k' was the rate of the transition in receptor affinity for the agonist. Estimates for k' were obtained from a linear least-squares fit of the data to the logarithmic form of eq. 1.

(3) Fluorescence Measurements

The equilibrium fluorescence of the AcChR-ethidium complex as a function of local anesthetic concentration was measured as previously described (Schimerlik and Raftery, 1976; Schimerlik *et al.*, 1979a) using a Perkin-Elmer MPF-4 spectrofluorimeter thermostated at 25° C. The excitation and emission wavelengths were 483 and 610 nm, respectively.

Kinetic data were obtained using a Durrum D-110 stopped-flow instrument equipped with a 75-W xenon lamp and operating in the fluorescence mode. Excitation was at 493 nm and fluorescence emission was monitored using a Corning 3-69 filter (50% transmission at 530 nm). The final concentration of membrane fragments after mixing was typically 0.5 μ M in α -BuTx sites and the temperature was maintained at $25 \pm 1^{\circ}$. The amplified photomultiplier output was collected using a Biomation Model 805 transient recorder connected to a Digital Equipment Corporation MINC computer and a Tektronix 5103N storage oscilloscope, the latter being used only for visual monitoring of the data.

All fluorescence data were analyzed by a non-linear regression method using BASIC programs written for the MINC computer. This method uses the algorithm of Marquardt (1963) and was adapted from Bevington (1969). For each trace both the raw data and the curve obtained using the parameters derived from the fitting procedure were plotted on a Hewlett-Packard 7004B X-Y recorder to aid in the assessment of the fit of the data to the chosen experimental model. Typically 128 evenly spaced points of the 2048 collected for each trace were used in data analysis.

Kinetic data were analyzed using either a single exponential equation

$$F(t) = A_0 + A_1 \exp(-k_1 t) + k_0 t$$

or a sum to two exponentials

$$F(t) = A_0 + A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + k_0 t$$

where $F(t)$ is the observed fluorescence at time t , A_0 is the equilibrium fluorescence level, A_1 and A_2 are the amplitudes of the two processes and k_1 and k_2 are the corresponding rate constants. The term $k_0 t$ was included to correct for any decrease due to photolysis which was linear with time.

(4) Materials

Lidocaine was purchased from Pfaltz and Bauer, Inc., procaine from Sigma Chemical Co., and dibucaine and tetracaine from ICN Pharmaceuticals. Unless otherwise indicated, the buffer used in all

experiments was Torpedo Ringers (250 mM NaCl, 5 mM KCl, 4 mM CaCl_2 , 2mM MgCl_2 , 0.02% NaN_3 buffered by either 5 mM TRIS or 20 mM HEPES, pH 7.4). All other materials and methods were as described in the previous chapter.

Results

(1) Effects of Local Anesthetics on the Agonist-Induced Increase in Ligand Affinity

In general, the local anesthetics tested inhibited [125 I]- α -BuTx binding only weakly, with inhibition constants in the millimolar range (Fig. 1). Only dibucaine showed a significant inhibition of toxin binding at lower concentrations ($K_I = 56 \pm 6 \mu\text{M}$; Fig. 1). At a concentration of 5 μM dibucaine did not significantly alter the rate of toxin binding, as predicted from its K_I . If, however, 1 μM carb was added simultaneously with the [125 I]- α -BuTx to membranes containing this concentration of dibucaine, the observed rate of toxin binding was slower than that observed in the presence of only carb or only dibucaine (Fig. 2A). Assuming competitive inhibition of toxin binding by both of these ligands, a decreased rate of [125 I]- α -BuTx binding would reflect an increased affinity of the AcChR for carb or dibucaine or for both compounds. The experiments described below allowed discrimination between these possibilities.

Membrane fragments were incubated with carb to induce conversion of the receptor to the high affinity state. When such membranes were diluted 40-fold into buffer containing dibucaine and toxin, the initial rate of [125 I]- α -BuTx-receptor complex formation was the same as that observed when the membranes were diluted into buffer containing only [125 I]- α -BuTx (Fig. 2B). If dibucaine association with the AcChR was in rapid equilibrium (with respect to toxin binding), this demonstrated that the affinity of the receptor for dibucaine was

FIGURE 1: Inhibition of Toxin Binding to Membrane Bound AcChR as a Function of Local Anesthetic Concentration: Membrane fragments 63 nM in toxin sites were incubated with local anesthetic for 20-30 minutes. The reaction was initiated by adding [125 I]- α -BuTx (final concentration, 520 nM) and the kinetics of toxin binding were followed to at least 50% completion. The apparent rate constants for toxin binding in the presence of ligand, $k_{(L)}$, were determined from linear least squares estimations of the slopes of logarithmic plots of the data as described in the Materials and Methods section.

(A) (● - ●) dibucaine and (○ - ○) tetracaine

(B) procaine (● - ●), and (○ --- ○) lidocaine.

The curves were drawn using apparent inhibition constants, (K_I 's, listed in Table 1) calculated from a weighted linear least squares fit to the equation $[k_{(L)}]^{-1} = [k_{(L=0)}]^{-1} (1 + L/K_I)$.

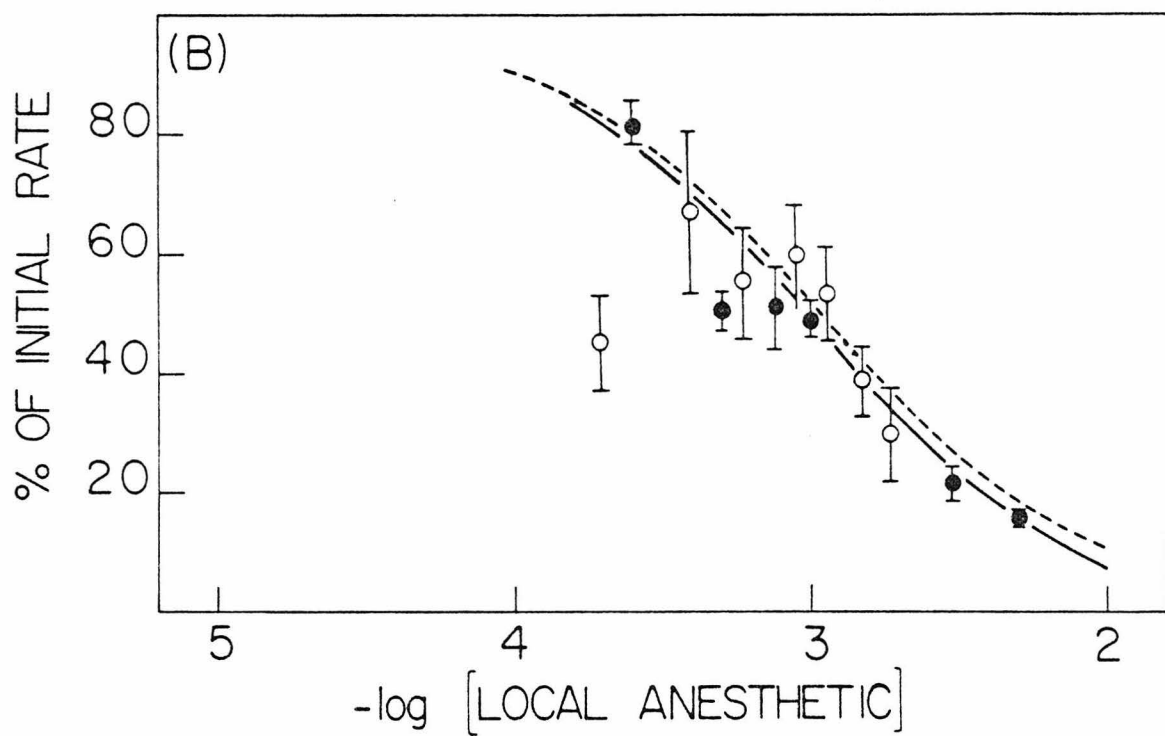
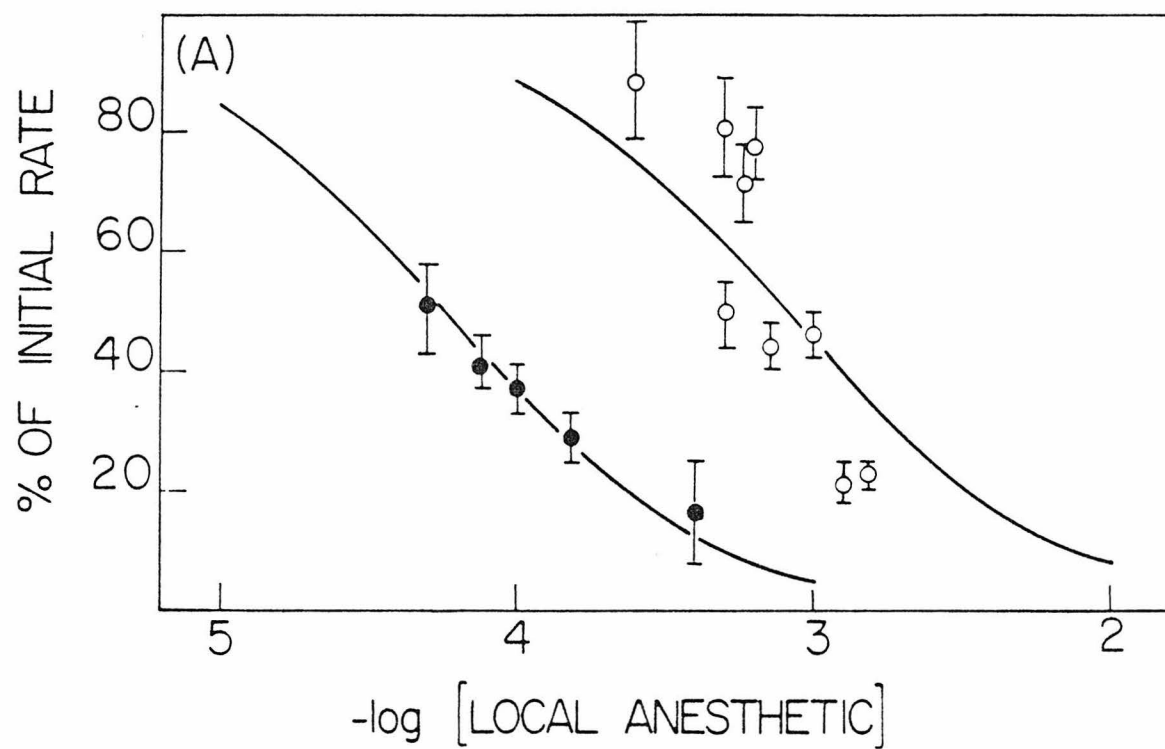


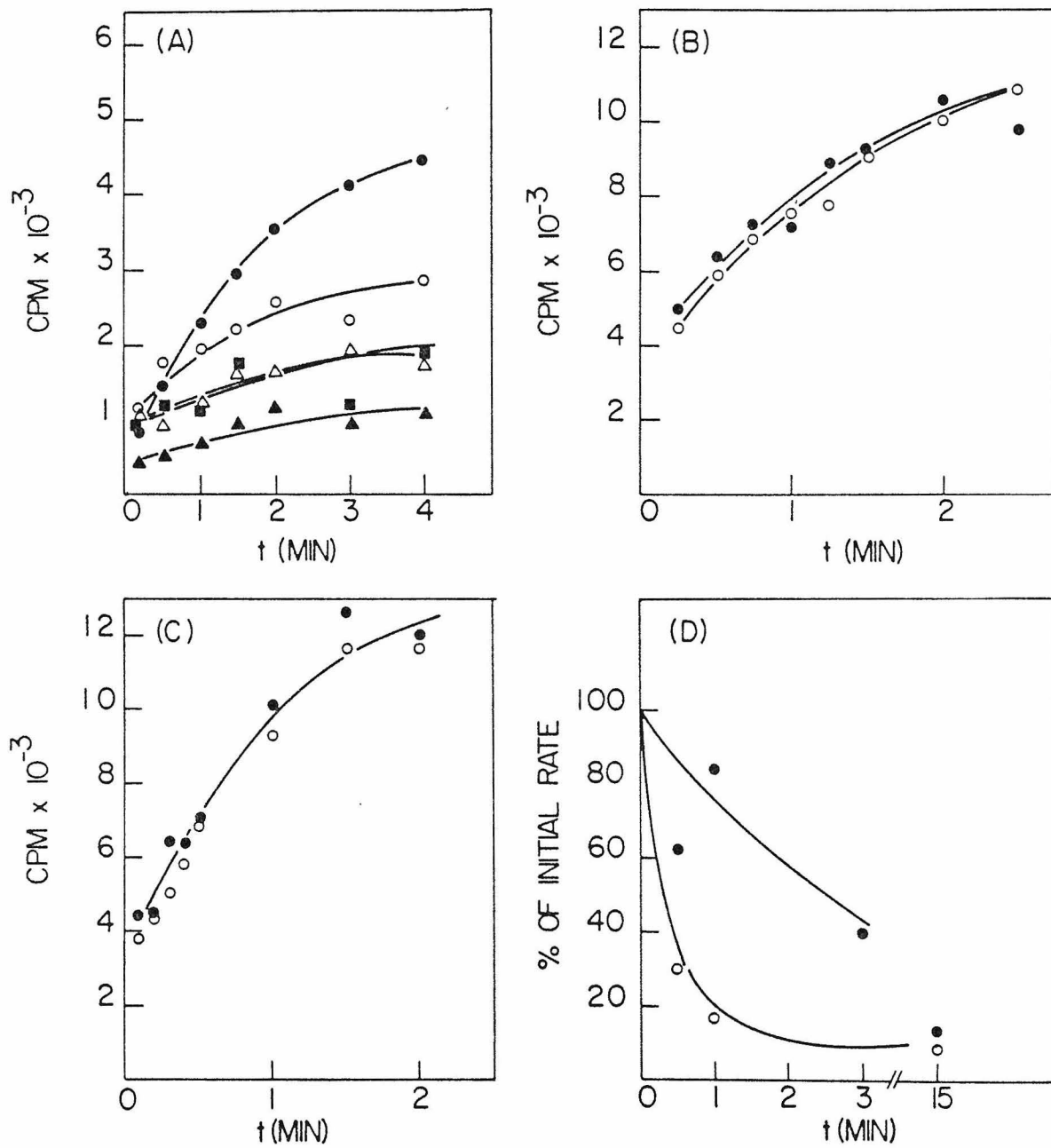
FIGURE 2: (A) Inhibition of [125 I]- α -BuTx Binding to AcChR Enriched Membranes by Carb in the Presence of Dibucaine: The formation of [125 I]- α -BuTx-AcChR complex was followed as described in Materials and Methods. The concentrations were 30 nM in toxin sites, 240 nM in [125 I]- α -BuTx, 1 μ M in carb and 5 μ M in dibucaine. (●) [125 I]- α -BuTx binding to membrane fragments; (○) carb and [125 I]- α -BuTx added simultaneously to membrane fragments; (▲) membrane fragments were incubated with carb for 15 minutes before the addition of [125 I]- α -BuTx; (Δ) dibucaine, carb and [125 I]- α -BuTx were added to membrane fragments simultaneously; (■) membrane fragments were incubated 15 min with dibucaine followed by addition of carb and [125 I]- α -BuTx.

(B) [125 I]- α -BuTx Binding to Membranes in the High Affinity State for Agonists in the Presence of Dibucaine: Membrane fragments (2.6 μ M in toxin sites) were incubated with 1 μ M carb for 45 minutes at room temperature to convert the receptor to the high affinity state. Aliquots were diluted 40-fold into Torpedo Ringers containing [125 I]- α -BuTx (470 nM) without (●) or with (○) 5 μ M dibucaine.

(C) Binding of [125 I]- α -BuTx to AcChR Enriched Membranes Following Prolonged Exposure to Dibucaine: (○) membrane fragments 1.6 μ M in [125 I]- α -BuTx sites were incubated for two hours at 4° with 0.83 μ M dibucaine and then diluted (20-fold) into Torpedo Ringers containing 1 μ M carb and 0.47 μ M [125 I]- α -BuTx, and formation of toxin-receptor complex was monitored. (●) Membranes incubated for 2 hours in Torpedo Ringers only, and then assayed as described above.

(D) Increase in the Rate of the Carb Induced Affinity Change in the Presence of Dibucaine: AcChR enriched membrane fragments (87 nM in

[¹²⁵I]- α -BuTx sites) were incubated for 5 minutes without (●) or with (○) 1 μ M dibucaine. Carb was added (final concentration, 1 μ M), and the membranes were incubated for the indicated times before [¹²⁵I]- α -BuTx (final concentration, 0.51 μ M) was added and the rate of [¹²⁵I]- α -BuTx AcChR complex formation was followed as described in the Materials and Methods. Plots show the apparent rate of toxin binding, k_{app} , versus time of incubation with carb. The solid lines were calculated using apparent rate constants for the affinity transition of $k' = 0.010 \pm 0.002 \text{ s}^{-1}$ in the absence of dibucaine, in agreement with previously reported values (Lee et al., 1977; Quast et al., 1978), and $k' = 0.026 \pm 0.008 \text{ s}^{-1}$ in the presence of dibucaine. These values were obtained from weighted linear least squares fits to the logarithmic form of eq. 1.



independent of the affinity state (high or low) for agonists. It could be deduced then that dibucaine increased the affinity of the AcChR for carb.

If the high affinity state for agonists in the presence of carb and dibucaine was the same as that state induced by carb alone, there are two possible explanations for this result. The first is that dibucaine induced the change in affinity, but its affinities for the two states did not differ appreciably. This type of behavior has been observed for both hexamethonium and d-tubocurarine (Quast *et al.*, 1978). In order to test this possibility membrane fragments were incubated with dibucaine for two hours and the rate of toxin binding was measured after 40-fold dilution into buffer containing 1 μ M carb and [125 I]- α -BuTx. As shown in Fig. 2C, the rate did not differ from that of a control which had not been exposed to dibucaine. Thus, the local anesthetic alone did not cause conversion of the AcChR from a state of low to one of high affinity. The second possibility was therefore considered, i.e., that dibucaine modified the rate of the carb-induced time-dependent affinity change. The data shown in Fig. 2D demonstrate that dibucaine did indeed greatly increase the rate of this transition.

The effects of several other compounds on the rate of the carb-induced increase in affinity were tested. In all cases the concentration of anesthetic used was well below the K_I determined by [125 I]- α -BuTx inhibition so that a direct inhibition of toxin binding by the compound being tested could be ruled out. The results of these studies are summarized in Table 1. The local anesthetic tetracaine presented an especially interesting case. The data in Fig. 3 can be interpreted as a demonstration that, at 50 μ M, tetracaine decreased the rate of the

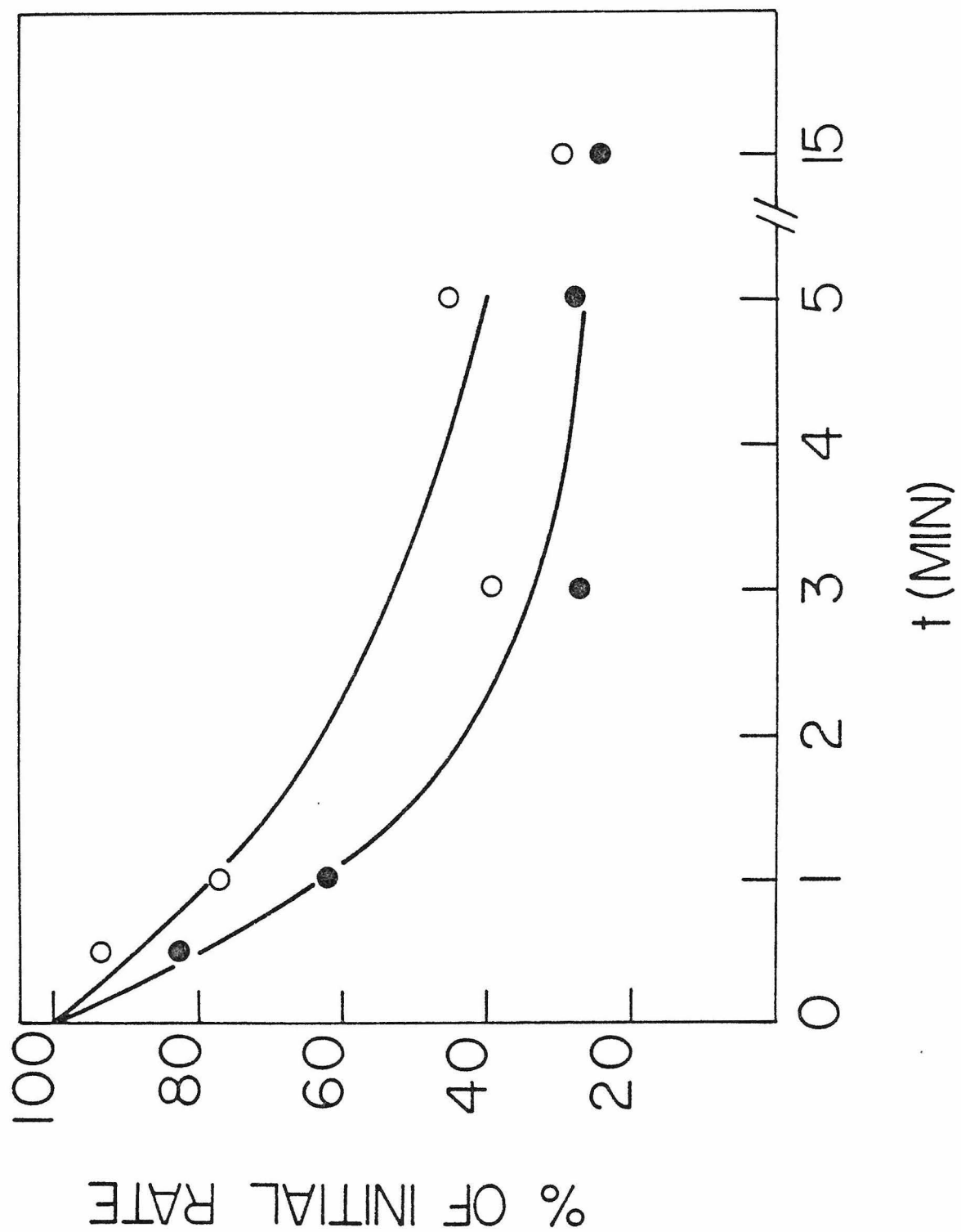
Table I: Effects of Local Anesthetics on Properties of AcChR Membrane Fragments

Local Anesthetic	Inhibition of [¹²⁵ I]- α -BuTx Binding K _I , (μ M)	Displacement of [³ H]H ₁₂ -HTX specifically bound K _I , (μ M) ²		Effect on Rate of Carb Induced Affinity Change	
		(-)-carb	(+)-carb		effect ¹
				Concentration Tested (μ M)	
dibucaine	56 $\pm$ 6	80	7.5	++	1-5
tetracaine	800 $\pm$ 100	1.5	30	-	50
lidocaine	1100 $\pm$ 200	2500	600	+	150
procaine	930 $\pm$ 60	3000	3000	0	150

1. "+" = increase rate; "-" decrease rate; "0" = no effect.

2. Data taken from Blanchard et al., 1979a.

FIGURE 3: Decreased Rate of the Carb-Induced Affinity Change in the Presence of Tetracaine: The experimental details were the same as in Fig. 2D except that 50 μ M tetracaine was used in place of dibucaine. The calculated rates for the carb induced increase in affinity were $k' = 0.012 \pm 0.003 \text{ s}^{-1}$ (●) in the absence of tetracaine and $k' = 0.006 \pm 0.002 \text{ s}^{-1}$ (○) in the presence of 50 μ M tetracaine.



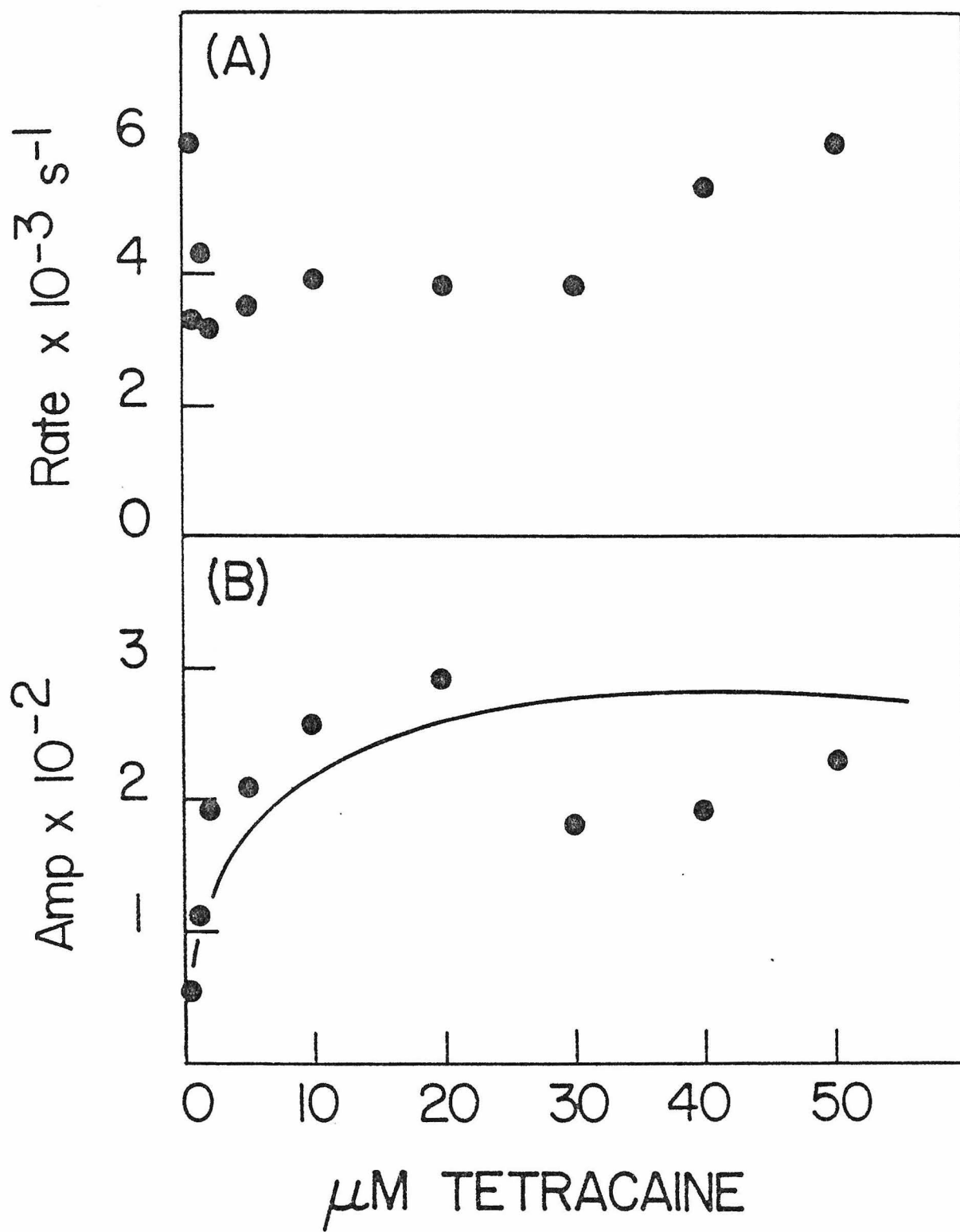
low to high affinity transition caused by carb. This result was in contrast to those obtained with the other compounds which either increased or did not affect the rate of this process under similar conditions.

(2) Kinetics of Tetracaine Binding using Ethidium as Indicator

Upon rapid mixing of tetracaine and AcChR-enriched membrane fragment solutions a slow decrease in the fluorescence intensity of membrane-associated ethidium was observed. This signal could be fitted well to a single exponential equation. As shown in Fig. 4A the rate of this process was virtually independent of tetracaine concentration over the range studied and was about 0.004 s^{-1} . The amplitude of the fluorescence decay, however, saturated with increasing tetracaine concentration reaching a half-maximal value at about $1 \text{ } \mu\text{M}$ tetracaine (Fig. 4B).

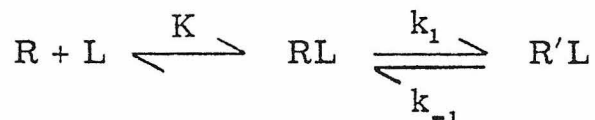
The observed kinetic signal could not be due to a simple binding of tetracaine to the AcChR-Eth complex since this process would be expected to be much faster than that observed. Therefore, the kinetic signal could be attributed to a ligand-induced conformational change. In order to distinguish between possible mechanisms for tetracaine binding it would be necessary to measure the rate of the kinetic process at tetracaine concentrations where it was not yet saturated. Unfortunately, accurate experimental data in this concentration range could not be obtained due to the low amplitude of the signal. Two mechanisms consistent with the available data are presented in the discussion.

FIGURE 4: Effect of Tetracaine Concentration on the Kinetics of Binding to AcChR: Equal volumes of membrane fragments ($0.5\ \mu\text{M}$ in $\alpha\text{-BuTx}$ sites final concentration) and tetracaine solution were mixed in the stopped-flow instrument and the resulting fluorescence signal was recorded. The concentration of Eth was $2\ \mu\text{M}$ in both syringes and all data points are an average of two to four determinations. The rate, (A), and amplitude, (B), of the observed signal is shown. The solid line in (B) has no theoretical significance.



(3) The Kinetics of Binding of Other Local Anesthetics to the AcChR

When low concentrations of the local anesthetic dibucaine were mixed rapidly with AcChR membrane fragments in the presence of Eth a biphasic kinetic pattern consisting of an initial fluorescence decrease followed by a slower increase was observed (Fig. 5A). At higher concentrations of local anesthetics the amplitude of the slower phase (the increase) was negligible (Fig. 5B). The rate of the fast fluorescence decrease (Fig. 6A) increased with increasing dibucaine concentration, reaching a limiting value of $\sim 0.13 \text{ s}^{-1}$ at concentrations above $\sim 75 \mu\text{M}$. The amplitude of this phase (Fig. 6B) also saturated at high dibucaine concentrations with a midpoint of $\sim 5 \mu\text{M}$. The data for this phase are consistent with a mechanism involving the binding of a ligand molecule (L) to the receptor (R) followed by a conformational change of the RL complex



where K is the dissociation constant for the initial binding and k_1 and k_{-1} are rate constants for the (slow) conformational change. However, because there was no evidence that the two phases (fast decrease and slow increase in fluorescence) were kinetically uncoupled, it was necessary to consider more complicated models that predicted two relaxation times.

Figure 6C shows that the rate of the slow fluorescence increase for dibucaine was linear over the concentration range examined. The

FIGURE 5: Kinetics of Dibucaine Binding to AcChR Membrane

Fragments: Stopped-flow-fluorescence traces obtained after mixing AcChR membrane fragments with dibucaine are shown. Final concentrations after mixing were 0.5 μM in α -toxin sites, 2 μM Eth, and dibucaine = 3 μM (A) or 30 μM (B). The solid lines were calculated from the parameters (given below) obtained from the best fit to the data as described in methods. (A) $k_1 = 0.032 \text{ s}^{-1}$; $A_1 = -708 \text{ mV}$; $k_2 = 0.007 \text{ s}^{-1}$; $A_2 = 476 \text{ mV}$. (B) $k_1 = 0.081 \text{ s}^{-1}$; $A_1 = -1030 \text{ mV}$; $k_2 = 0.014 \text{ s}^{-1}$; $A_2 = 95 \text{ mV}$.

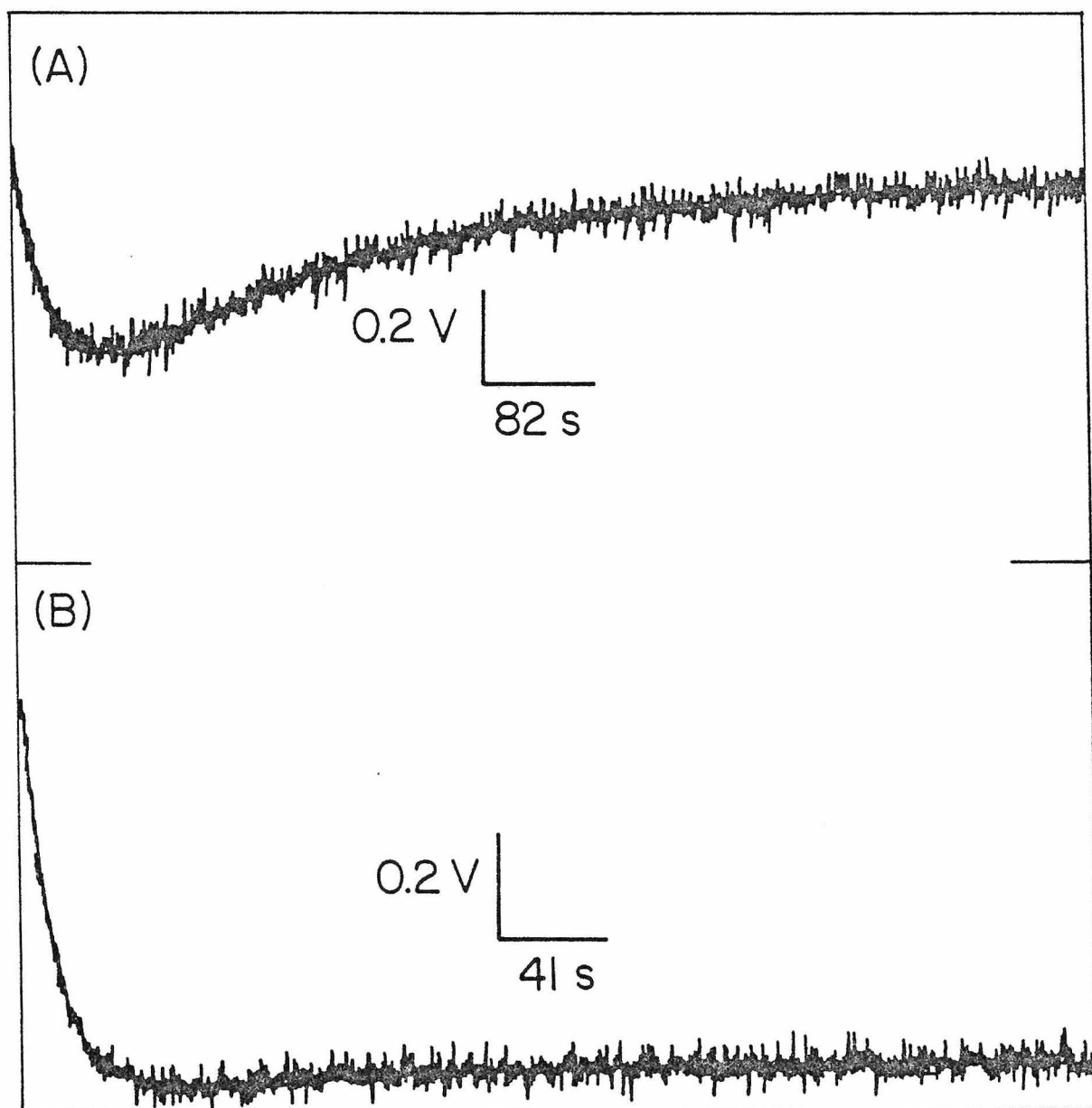
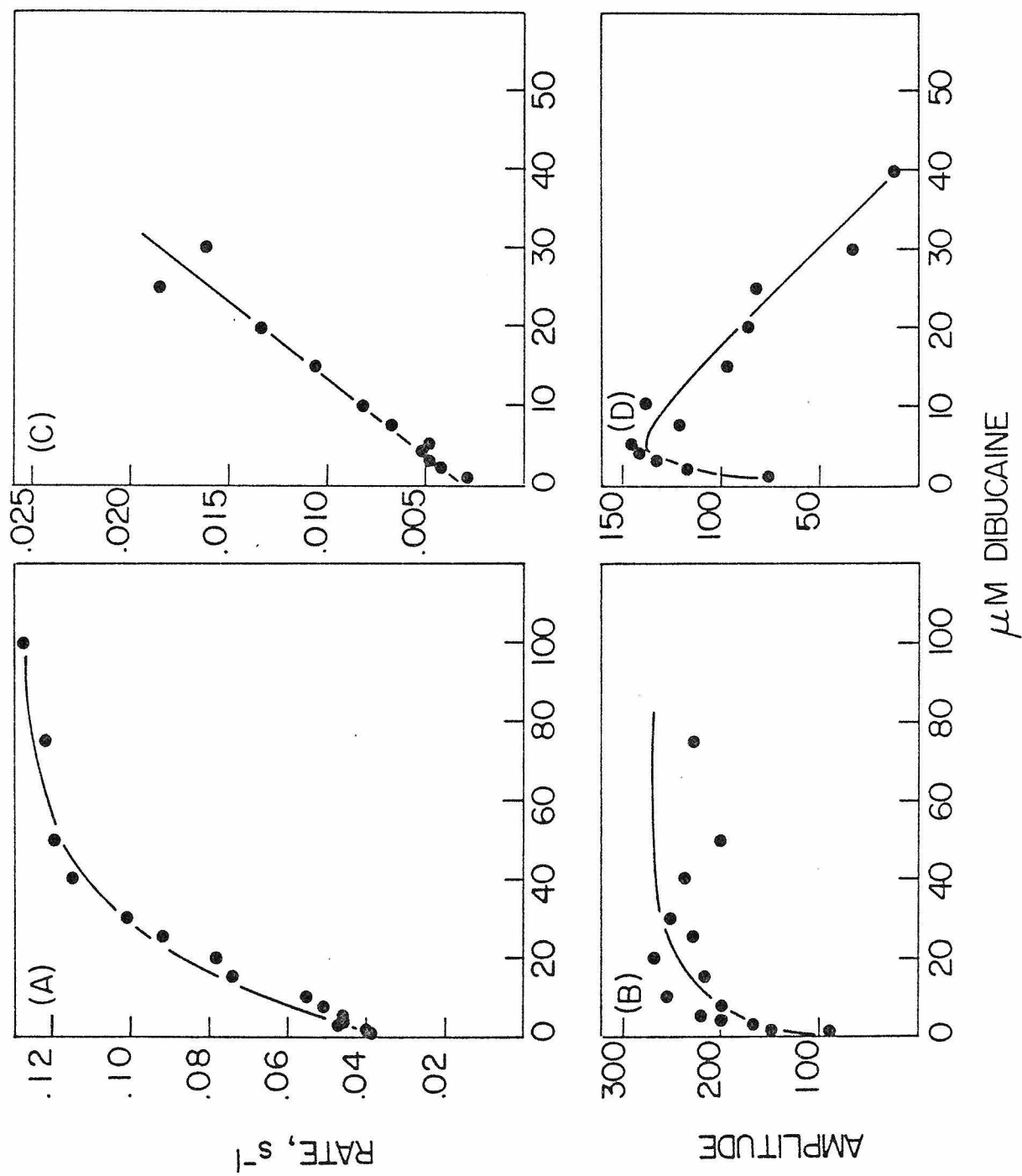


FIGURE 6: Kinetic Parameters as a Function of Dibucaine

Concentration: The dependence of the rate and amplitude (in arbitrary units), respectively for the fast fluorescence decrease (A,B) and the slow fluorescence increase (C,D) observed on mixing membrane fragments with dibucaine are shown. Final concentrations of AcChR and Eth were 0.5 and 2 μ M, respectively. Each point is the average of at least two determinations. The solid lines were drawn by hand.



amplitudes of this phase (Fig. 6D) increased to a maximum at a concentration of $\sim 5 \mu\text{M}$ and decreased toward zero at higher concentrations. As shown in Fig. 7 the concentration dependence of the kinetics of lidocaine binding was very similar (though shifted to higher ligand concentrations) to that observed for dibucaine (Fig. 6). The fluorescence signal obtained on rapid mixing of procaine with AcChR-enriched membrane fragments also consisted of two phases. Although the slow increase in fluorescence intensity showed a concentration dependence similar to those seen in Figs. 6 and 7 (C,D) the fast decrease in fluorescence was of small amplitude so that an accurate determination of the variations of its rate and amplitude with concentration could not be made.

The fluorescence signals seen on binding of local anesthetics to the AcChR-Eth complex could be the result of either a change in the quantum yield of bound dye (reflecting a change in the conformation of AcChR-Eth complex) or of a change (uptake and/or release) in the amount of bound Eth. Schimerlik *et al.* (1979a) showed that addition of local anesthetics to AcChR-enriched membrane fragments did not alter the amount of [^3H] Eth bound. However, an uptake or release of bound Eth could not be ruled out since (a) the equilibrium studies did not eliminate the possibility of a transient change in the concentration of bound Eth and (b) a large change in the fluorescence intensity might result from only a small change in the concentration of bound indicator. Therefore, the effect of Eth concentration on the kinetics of dibucaine binding to the receptor was investigated. Figure 8A shows that the rate of the initial decrease in fluorescence intensity was independent of

FIGURE 7: Kinetic Parameters as a Function of Lidocaine

Concentration: The conditions were as described for Fig. 6 except that the concentration dependence for the local anesthetic lidocaine is shown. The rates (A, C) and amplitudes (B, D) of the fast and slow fluorescence processes, respectively are plotted.

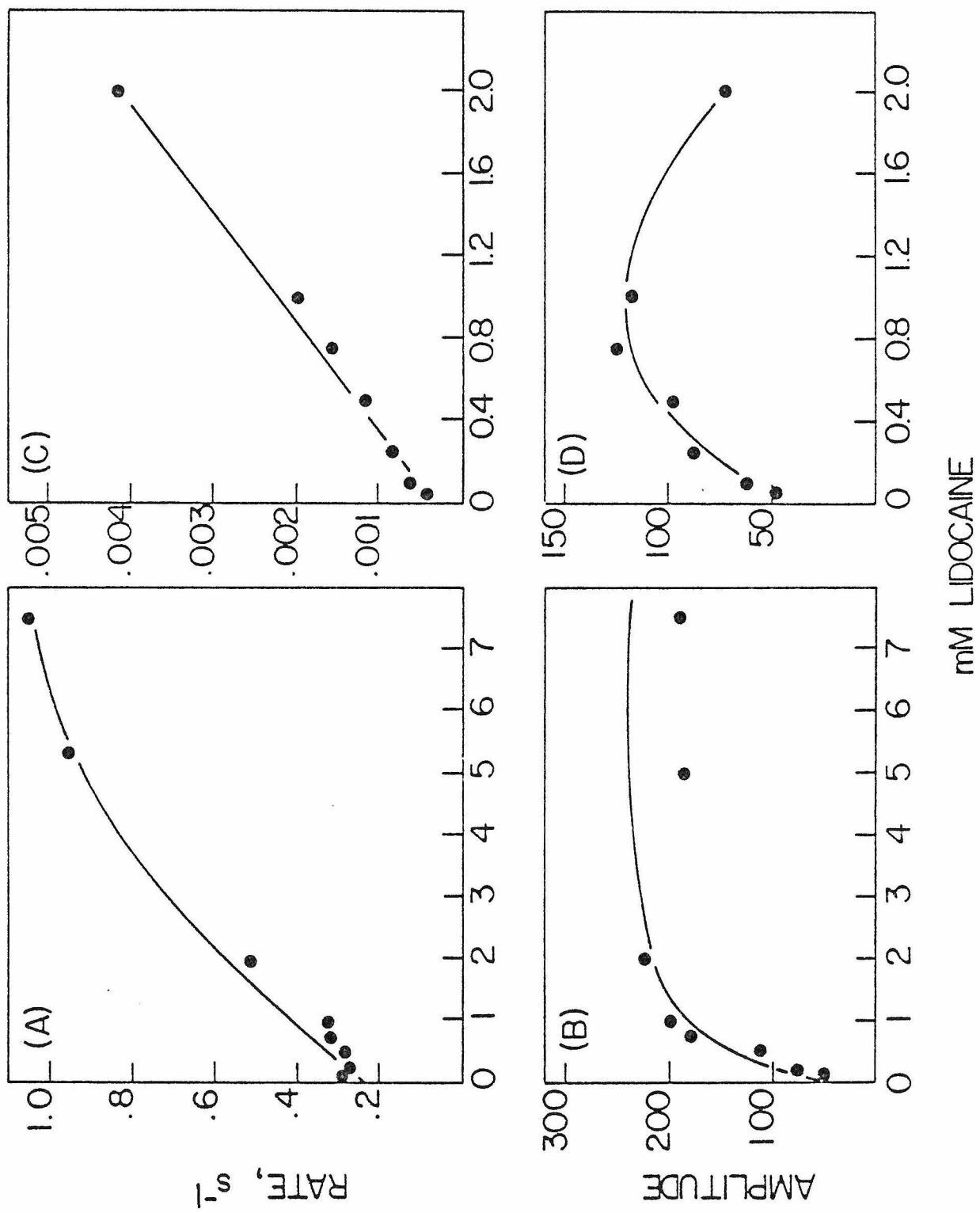
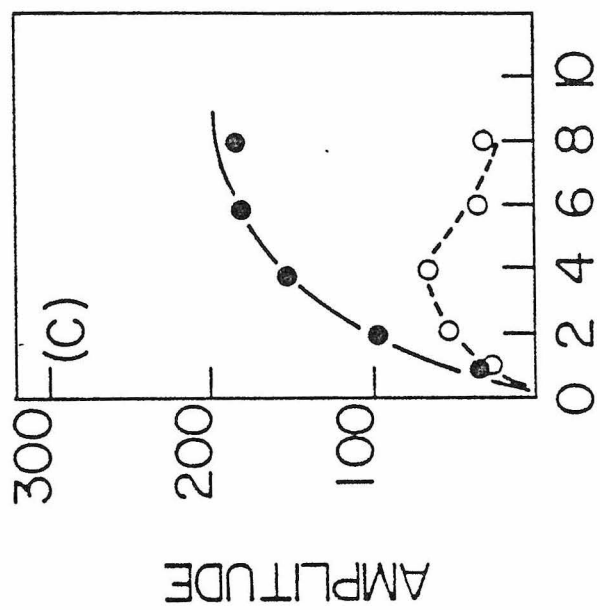
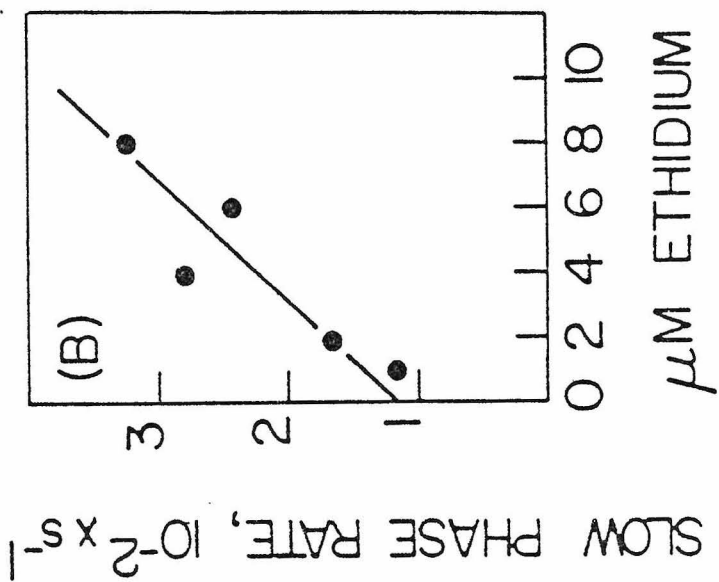
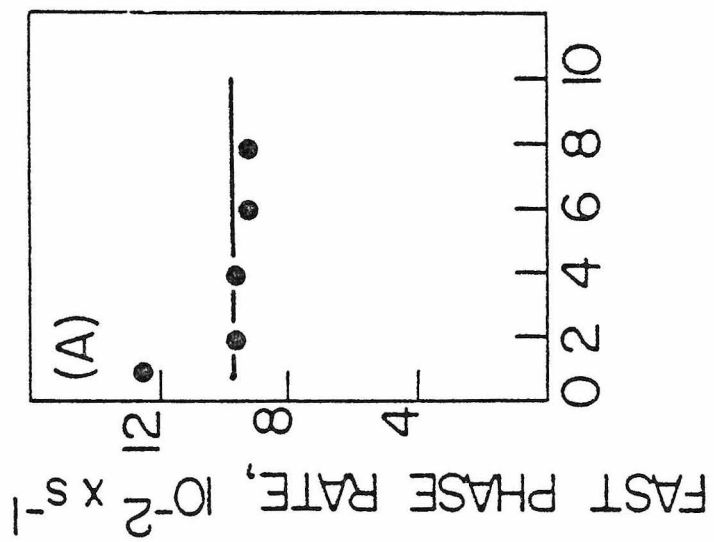


FIGURE 8: Dependence on the Kinetics of Dibucaine Binding on Ethidium Concentration: The final concentrations were: dibucaine = $10\ \mu\text{M}$ and AcChR = $0.5\ \mu\text{M}$. All data points are the average of two determinations. The lines drawn through the points have no theoretical significance. The rates of the rapid decrease (A) and the slow increase (B) in fluorescence intensity as a function of Eth concentration are shown. (C) shows the amplitudes of the fast ($\bullet\text{---}\bullet$) and slow ($\circ\text{--}\text{--}\circ$) processes, respectively.



ethidium concentration over the region studied. The amplitude of this phase (Fig. 8c) reached a maximal value with increasing Eth concentration, indicating the existence of a saturable population of Eth binding sites. The fast phase then, was most likely due to a change in the quantum yield of specifically bound Eth as a result of a conformational change in the AcChR.

The rate of the slow phase (Fig. 8B) increased linearly as the Eth concentration was raised. This dependence, though fairly small, indicated that the slow increase in fluorescence intensity was possibly due to an increase in the amount of bound Eth. The decrease in the amplitude of the slow phase (Fig. 8C) at high concentrations of Eth could be attributed to an uptake of specifically bound dye. At high concentrations all of the specific binding sites would be occupied so that no further dye could bind. Large amounts of Eth are non-specifically bound to the membrane fragments and the quantum yields of the free, non-specifically bound and specifically bound dye are all different (Schimerlik et al., 1979a). Therefore, we could not evaluate possible mechanisms for local anesthetic binding because the change in the concentration of bound ethidium during the slow kinetic phase could not be determined.

Discussion

The local anesthetics inhibited the binding of [^{125}I]- α -BuTx only weakly, in agreement with results reported earlier by other investigators (Weber and Changeux, 1974; Weiland et al., 1977). Local anesthetics have also been reported to displace [^3H]H₁₂-HTX specifically bound to AcChR-enriched membrane fragments (Blanchard et al., 1979). The apparent inhibition constants determined in the presence and absence of 10 μM carb are listed in Table 1. It can be seen that (with the exception of tetracaine) the K_{I} 's for direct inhibition of [^{125}I]- α -BuTx binding and for the displacement of specifically bound [^3H]H₁₂-HTX in the absence of carb are similar for a given anesthetic. However, it should be noted that HTX and α -BuTx bind to different sites (Elliott and Raftery, 1977). In addition, with membranes in the high affinity state for agonist, dibucaine displaced [^3H]H₁₂-HTX at a concentration ten-fold lower than it inhibited [^{125}I]- α -BuTx binding (Table 1). If dibucaine bound to a single site both inhibited the binding of [^{125}I]- α -BuTx and caused the displacement of [^3H]H₁₂-HTX then the apparent K_{I} values for these two effects on high affinity membranes should be equal. It was therefore concluded that the sites of local anesthetic interaction responsible for inhibition of [^{125}I]- α -BuTx binding and the displacement of specifically bound [^3H]H₁₂-HTX were distinct.

Both local anesthetics (Weiland et al., 1977; Briley and Changeux, 1978) and general anesthetics (Young et al., 1978) have been found to affect the agonist-induced increase in ligand affinity of Torpedo membranes at concentrations below those that directly inhibit the

binding of α -toxins to the AcChR. We have observed that the magnitude of this effect depends on the compound being tested; whereas the local anesthetics dibucaine and lidocaine caused an increase in the rate of the carb-induced affinity change (Fig. 2D and Table 1), both procaine (Table 1) and procaine amide azide (Chapter 3) showed little effect on the rate of this transition. The most interesting case, however, was that of tetracaine (Fig. 3). The rate of the carb-induced affinity change was slower in the presence of this compound than in its absence. In contrast, Weiland, et al. (1977) reported that local anesthetics facilitated the conversion of the receptor to a high affinity state. However, the data reported by these authors were obtained in the absence of calcium while the results reported here were performed in Torpedo Ringers buffer (containing 4mM calcium). Since calcium has been shown to increase the rate of the affinity change for cholinergic ligands in vitro (Lee et al., 1977) the results of these two studies cannot be directly compared.

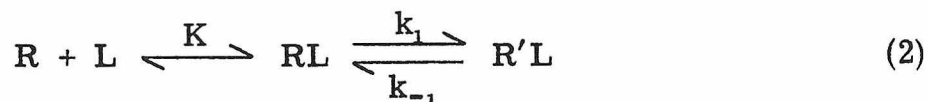
As can be seen in Table 1, the effects of local anesthetics on the rate of the carb-induced increase in receptor affinity for agonists roughly paralleled the differences in their apparent affinities for the low and high affinity states of the receptor as reflected by [^3H]H₁₂-HTX displacement (in the absence and presence of 10 μM carb, respectively). A possible explanation for this parallel was that the rate of the agonist-induced transition in receptor affinity was modulated by compounds bound to the [^3H]H₁₂-HTX binding site with the magnitude of the effect determined by the ratio of the K_I's to the two states: with compounds such as dibucaine that bind more tightly to the high affinity state causing

an increased rate of transition and tetracaine, which binds more tightly to the low affinity state, decreasing the rate of the transition. This explanation is also consistent with the observation that compounds such as HTX (Elliott and Raftery, 1977) and PAA (Chapter 3), that did not appreciably change the rate of the receptor transition also showed little or no difference in their apparent affinities for the low and high affinity states.

Another possible explanation of the effects of local anesthetics on the carb-induced increase in AcChR affinity is that these compounds perturb the resting conformation of the receptor in a nonspecific manner such as disturbing the lipid annulus surrounding the receptor. Such a mechanism has been previously suggested as a possible explanation of the effects of both local (Weiland et al., 1977) and general anesthetics (Young et al., 1978) on membrane bound AcChR from Torpedo, as well as of the effects of anesthetics on proteins in general (Richards et al., 1978). The fact that local anesthetics displace specifically bound [^3H]H₁₂-HTX does not rule out this explanation: following dissolution of AcChR enriched membranes in the nonionic detergent Triton X-100 both the ability to undergo the agonist mediated transition in affinity (V. Witzemann and M. Raftery, unpublished) and specific [^3H]H₁₂-HTX binding (Elliott and Raftery, 1977) were lost. In addition, 2% (w/v) sodium cholate extracts of AcChR enriched membranes from Torpedo californica showed no specific binding of [^3H]H₁₂-HTX (Elliott and Raftery, 1977) but following dilution of the detergent to 0.5% (w/v) binding similar to that seen for intact membranes was recovered

(Elliott and Raftery, 1979). Considering the sensitivity of the specific binding of [^3H]H₁₂-HTX to perturbations of the membrane environment of the AcChR, binding of local anesthetics to sites other than the specific [^3H]H₁₂-HTX binding site might result in displacement of specifically bound HTX by means of changes in receptor conformation. As an example, Triton X-100 displaced bound [^3H]H₁₂-HTX at concentrations in the range of 0.005% (w/v), which are well below (approximately 20-fold) those at which the membranes begin to dissolve (Raftery *et al.*, 1973), and the displacement curve was shifted to lower concentrations in the presence of carb (Elliott, 1979). Therefore, the parallel observed between the effects of local anesthetics on [^3H]H₁₂-HTX displacement and on the rate of the low to high affinity transition (for agonists) might be expected even if these effects were the result of nonspecific perturbation of the membranes by local anesthetics.

The amplitude of the fluorescence decrease observed on binding of tetracaine to AcChR-Eth complex appeared to be a simple titration curve with a midpoint of $\sim 1 \mu\text{M}$ (Fig. 4B). This value was in good agreement with the inhibition constant of $1.5 \mu\text{M}$ determined by displacement of radiolabeled H₁₂-HTX (Table 1). Because H₁₂-HTX and tetracaine appear to compete for a common binding site (Blanchard *et al.*, 1979) it was possible that the binding of tetracaine to the AcChR could be described by the model for HTX binding proposed by Schimerlik *et al.* (1979b). This model may be written as:



where ligand (L) is considered to be in rapid equilibrium with receptor (R) with a dissociation constant, K. Ligand binding is followed by a slow conformational change to the final complex R'L with forward and reverse isomerization constants of k_1 and k_{-1} , respectively. The relaxation rate for this mechanism is given by (Hammes and Wu, 1974):

$$\lambda = k_{-1} + k_1 L / (L + K) . \quad (3)$$

Assuming that the change in quantum yield occurs in the isomerization step the amplitude is:

$$A = QR_0 L / (L + KK_1) \quad (4)$$

where Q is the quantum yield, R_0 the total concentration of binding sites and $K_1 = k_{-1}/k_1$. From the data in Fig. 4B one obtains $K_1K \sim 1 \mu\text{M}$ and the rate dependence of Fig. 4A gives $K \ll 1 \mu\text{M}$ since no variation of λ with ligand concentration is observed. This implies that $K_1 \gg 1$ (i.e., $k_{-1} \gg k_1$) and that therefore very little R'L is formed at equilibrium. An alternative mechanism is discussed below.

A possible explanation for the decreased rate of in vitro "desensitization" in the presence of tetracaine (Fig. 3) is that it binds preferentially to the low-affinity state of the AcChR. The ratio of the K_I values for displacement of [^3H]H₁₂-HTX in the presence and absence of carb (Table 1) also indicate a stabilization of the receptor in a low-affinity state. This suggests the following mechanism for tetracaine binding:



In this case R and R' are considered to be the low and high affinity states of the receptor, respectively. K is the dissociation constant for tetracaine binding and the k_i are the rate constants for the transition between the two receptor states. The amplitude is given by eq. (4) while the relaxation rate is (Hammes and Wu, 1974):

$$\lambda = k_{-1} + k_1 K / (L + K) . \quad (6)$$

Again, examination of the data in Fig. 4 gives $K_1 \gg 1$. For this model therefore $R > R'$ in the absence of added ligand in agreement with the experimental observation that most receptor is in the low affinity state. However, the available data do not allow discrimination between this mechanism and the one described by eq. 2.

The binding of tetracaine to AcChR enriched membrane fragments as monitored by ethidium fluorescence could be fit to a single exponential decay. By contrast, the other local anesthetics tested showed a complex kinetic pattern consisting of a decrease in the fluorescence of bound ethidium followed by a slower increase in fluorescence intensity. Because the slow process involved an uptake of ethidium a quantitative analysis of the data was not possible. However, the concentration dependence of the fast phase was consistent with the binding of a ligand to receptor followed by a conformational change of the complex (Eq. 2). If the fast phase is assumed to reflect such a binding step then the overall dissociation constant for the reaction may be determined from the midpoint of the amplitude titration (see eq. 4). Figures 6B and 7B give values of K_{overall} of ~ 5 and $\sim 300 \mu\text{M}$ for dibucaine and lidocaine,

respectively. These values are approximately an order of magnitude lower than the inhibition constants for H_{12} -HTX displacement shown in Table 1. This suggests that some local anesthetics may bind to the AcChR at sites other than the HTX binding site, although others have been shown to be competitive with H_{12} -HTX (Neubig et al., 1979).

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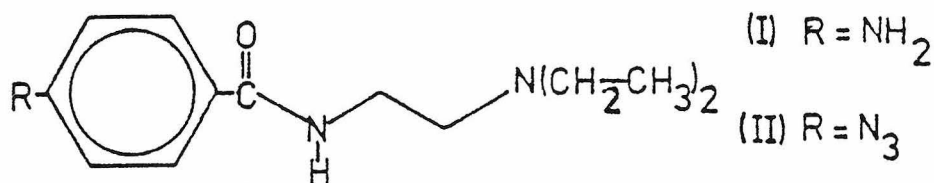
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CHAPTER 3: Identification of the Polypeptide Chains in Torpedo
Californica Electropex Membranes that Interact with a Local
Anesthetic Analog.

Introduction

AcChR enriched membrane fragments from Torpedo californica have been demonstrated to contain polypeptides of molecular weight 40,000, 50,000, 60,000, and 65,000 characteristic of the receptor purified by affinity chromatography (Duguid and Raftery, 1973; Reed et al., 1975) in addition to other major species with molecular weights of $\sim 43,000$ and $90,000$ (Witzemann and Raftery, 1977). Recently, it has been demonstrated that the $43,000$ and $90,000$ polypeptides are not required for normal receptor function (Moore et al., 1979) or for the binding of ligands to the AcChR (Neubig et al., 1979; Elliott et al., 1979; Chapter 1). Although the $40,000$ dalton polypeptide has been shown to be involved in the binding of α -toxins and cholinergic ligands the function of the $50,000$, $60,000$ and $65,000$ dalton subunits remain unclear (see Section 4 of the Introduction).

Because local anesthetics may inhibit receptor function by binding to and blocking the structures involved in translocation of ions across the postsynaptic membrane (Neher and Sakmann, 1976; Ruff, 1976; Steinbach, 1977) it was of interest to determine the sites of interaction of these compounds with AcChR-enriched membrane fragments. Therefore, we prepared an azido analog of the local anesthetic procaine amide (I); namely procaine amide azide (II). This compound was chosen instead of the procaine derivative (Levy



et al., 1977; Staros et al., 1978) because the amide bond is less susceptible to hydrolysis than is procaine's ester linkage. Photolysis of [^3H]-II in the presence of AcChR-rich membrane fragments was coupled with SDS/polyacrylamide gel electrophoresis to demonstrate which polypeptides of the membrane fragment preparation were involved in local anesthetic binding.

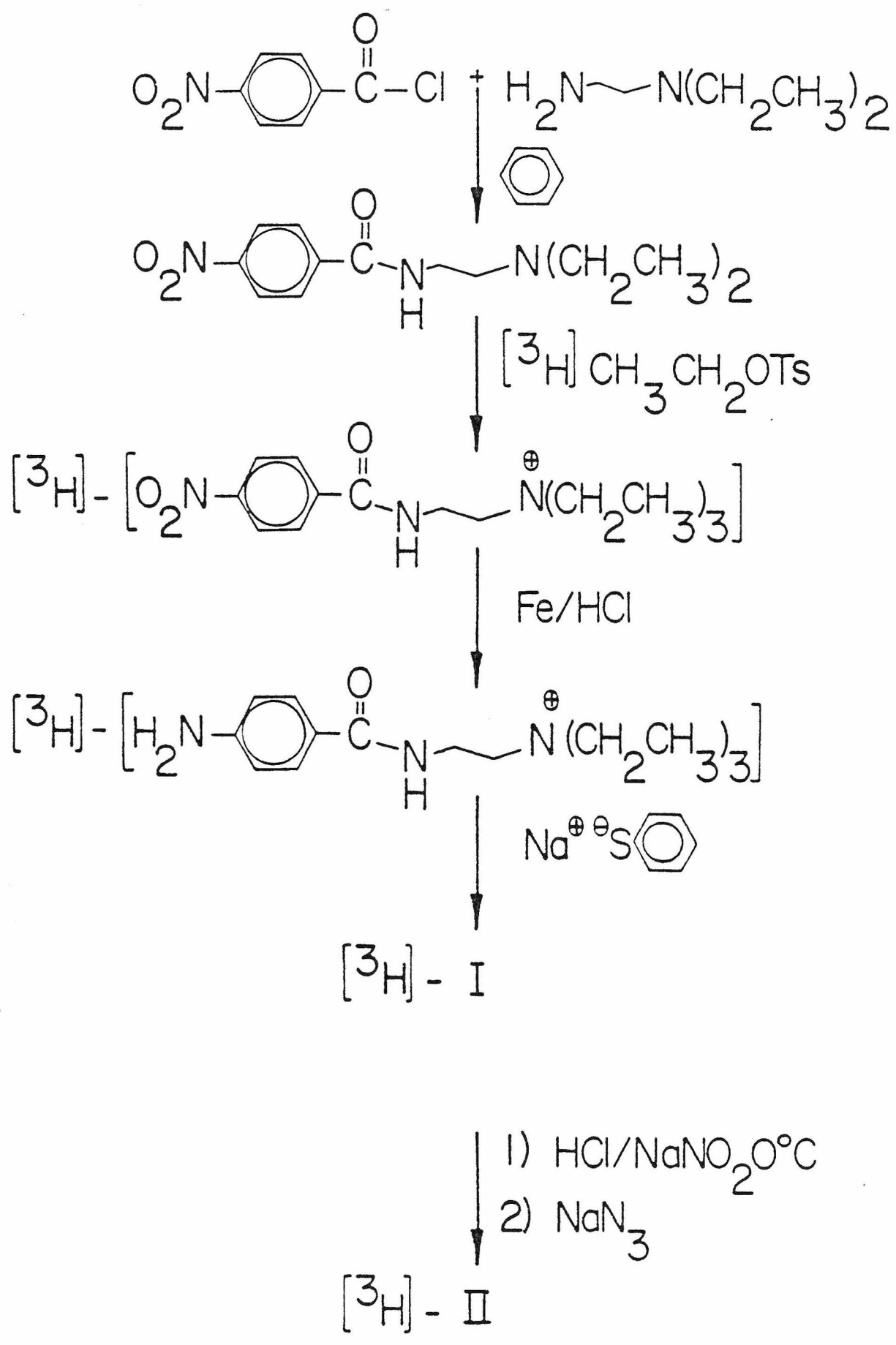
Materials and Methods

(1) Synthesis of Procaine Amide Azide

Unlabeled PAA was synthesized from procaine amide hydrochloride (Pfaltz and Bauer, Inc., Stamford, Conn.) by preparation of the corresponding diazonium salt followed by its reaction with sodium azide. The crude product was recrystallized from acetone: petroleum ether (30-60° fraction) and its identity was confirmed by elemental analysis. Calculated for $C_{13}H_{20}N_5ClO$: C, 52.4; H, 6.77; N, 23.5; Cl, 11.9. Found: C, 52.6; H, 6.8; N, 23.4; Cl 12.2.

The synthesis of [3H]-PAA is outlined in Fig. 1 and described in detail below. p-Nitrobenzoyl chloride (0.1 mole, Eastman) was dissolved in 400 ml dry benzene at 0° C and 0.1 mole N,N-diethylethylenediamine was added dropwise with stirring. After all of the amine was added the ice bath was removed and the mixture was stirred for two hours. The resulting crystals were collected by filtration and dissolved in ~800 ml H_2O . After filtration to remove insoluble material, the aqueous solution was brought to pH 12 and stored at 4° C. The 4-nitro-N-[2-(diethylamino)ethyl]benzamide separated as pale yellow crystals, M.P. 47-49° C (yield 5.4 g). [3H] ethyl tosylate was prepared from [$1-^3H$] ethanol (93.6 Ci/mol) using a four-fold molar excess of tosyl chloride in dry pyridine as described by Fieser and Fieser (1967). For the quaternarization step, 0.54 mmole [3H] ethyl tosylate and 0.43 mmole 4-nitro-N-[2-(diethylamino)ethyl] benzamide were dissolved in 0.5 ml redistilled tetramethylene sulfone and the solution was heated for 6 hr. on a steam bath. On addition of excess

FIGURE 1: Synthesis of [^3H] Procaine Amide Azide.



hot ether a white precipitate formed. The ether was discarded and the white powder was recrystallized from EtOH/EtOAc (yield: 0.2 g; 0.31 mmole). This product was dissolved in 5 ml 25% aqueous ethanol and 53 mg reduced iron and 0.33 ml of a solution of 0.05 ml concentrated HCl in 5 ml 25% aqueous ethanol was added. After refluxing for 1 hr., 10 μ l concentrated ammonium hydroxide was added and the mixture was filtered. A stream of dry Ar was used to remove solvent and the resulting oil was dried overnight in vacuo over P₂O₅. This product was subjected to the dequaternization reaction without further purification. Dequaternization to give [³H] procaine amide was accomplished by using thiophenoxide anion in 2-butanone (Shamma et al., 1966) as follows. The quaternary amine was dissolved in 12 ml freshly distilled 2-butanone under a nitrogen atmosphere and glacial acetic acid was added to a final concentration of 25 mM. Sodium thiophenoxide was added and the solution was refluxed for 48 hr, cooled, and 15 ml of 0.5 M citric acid was added. The solution was exhaustively extracted with petroleum ether and 15 ml of 2 M ammonium citrate/7.5 M ammonium hydroxide was added to the aqueous layer which was then extracted with five 25 ml portions of chloroform. The combined chloroform fractions were evaporated to dryness and the residue was identified as [³H] procaine amide by thin-layer chromatography on silica gel (Eastman # 13179) using 8:1 CHCl₃:MeOH as the solvent. In addition, when the above procedure was carried out using unlabeled ethyl tosylate, the product of the dequaternization step was positively identified as procaine amide by comparison of its proton magnetic resonance spectrum with that of an authentic sample. [³H] PAA was

prepared from the [^3H] procaine amide via the diazonium salt as described for unlabeled PAA.

The crude [^3H]-PAA was purified on a silica gel column using 8:1 CHCl_3 :MeOH. The fractions containing the peak of radioactivity were combined, the solvent was removed, and the residue was dissolved in water to give the stock [^3H] PAA solution. The concentration of the stock solution was calculated from its absorption at 270 nm and the specific activity of the [^3H]-PAA was determined by liquid scintillation counting of aliquots of the stock solution. Since there was a $\frac{2}{3}$ probability that a radioactive ethyl group would not be removed in the dequaternization reaction the expected specific activity of the product was $\frac{2}{3}$ that of the ethanol or 62.4 Ci/mol. The experimentally determined value of 54 Ci/mol $\pm \approx 10\%$ was therefore in good agreement with this predicted value.

(2) Labeling with [^3H] PAA and Polyacrylamide Gel Electrophoresis

Membrane fragments were incubated with the desired concentration of [^3H] PAA in the dark for 20-30 min at 0°C. The solutions were irradiated for 2 min in a quartz cuvette (with constant stirring) by using a UVSL-25 lamp (Ultraviolet Products, San Gabriel, CA) on the short-wavelength setting. The membranes were centrifuged for 15 min in an Eppendorf model 3200 centrifuge, and the supernatant was discarded. The pellet was resuspended in 1 ml of Torpedo Ringer's solution and the membranes were repelleted as above. After the supernatant was discarded, the pellets were dissolved in sample buffer [10% (wt/vol) glycerol/3% (wt/vol) SDS/100 mM dithiothreitol/62.5 mM

Tris-HCl, pH 6.8] and denatured by heating at 100° C for 2 min. SDS-polyacrylamide gel electrophoresis was as described in Chapter 1. Gels were either 1 × 10 cm or, when it was desired to distinguish between label on the 40,000 and 43,000 M_r bands 1 × 20 cm.

After electrophoresis the gels were sliced (either immediately or after staining) with a Hoeffer Scientific Instruments SL-280 gel slicer. Each slice was placed in a scintillation vial with 0.5 ml of 30% H₂O₂; the vials were tightly capped and heated at 70° C for 5 hr. After cooling and addition of scintillation fluid, the radioactivity in the vials was determined by using the tritium channel of a Packard 3375 liquid scintillation counter. For the 20-cm gels, radioactivity was measured in only that portion of the gel containing the 40,000-90,000 dalton bands since initial experiments showed no significant incorporation of radioactivity outside this molecular weight region.

For experiments using solubilized receptor, the buffer was 50 mM NaCl/10 mM sodium phosphate, pH 7.4/0.1% Triton X-100/0.02% NaN₃ and receptor solutions were concentrated by ultrafiltration (Amnicon CF50A membrane) before addition of sample buffer.

(3) Flux Experiments

The inhibition of agonist-mediated efflux of ²²Na⁺ by PAA was determined by the method of Miller et al. (1978).

(4) Miscellaneous

[³H] Ethanol was from New England Nuclear and sodium thiophenoxide was prepared by the method of Jenden and Hanin (1974).

Solubilized receptor was purified by affinity chromatography as described by Vandlen et al. (1976). All other experimental details are given in Chapters 1 and 2.

Results

(1) Characterization of the Effects of PAA on AcChR

PAA only weakly inhibited [^{125}I]- α -BuTx binding to membrane bound AcChR but was slightly more effective than its parent compound in this respect (Fig. 2A). Titration of membrane fragments with PAA by using the fluorescent probe ethidium (Fig. 2B) showed the same qualitative effects as seen with the local anesthetic procaine (Schimerlik and Raftery, 1976; Schimerlik *et al.*, 1979). When the data were corrected for nonspecific (i.e., not displaceable by α -BuTx) effects, a fluorescence increase was observed in the micromolar range, whereas addition of millimolar concentrations of PAA caused a decrease in the signal (Fig. 2B).

Figure 3 shows that PAA inhibited the carb-induced release of vesicle-entrapped $^{22}\text{Na}^+$. This demonstrated that PAA acted as a local anesthetic *in vitro*. 50 μM PAA was as effective in inhibiting $^{22}\text{Na}^+$ efflux as was a concentration of 500 μM (compare Figs. 3B and C) while 5 μM PAA caused only a slight inhibition of the flux response (not shown). Because inhibition of $^{22}\text{Na}^+$ efflux occurred at concentrations well below those which inhibited the binding of [^{125}I]- α -BuTx (Fig. 2A), it seemed likely that PAA could bind to (at least) two sites on AcChR membrane fragments: one site competitive with α -BuTx and the other related to the process of ion translocation.

Since local anesthetics have been reported to modify the rate of the carb-induced transition in receptor affinity at concentrations below those those that directly inhibit α -BuTx binding (Weiland *et al.*, 1977;

FIGURE 2: (A) Inhibition of initial rate of [125 I] α -BuTx binding as a function of ligand concentration: Membrane fragments 63 nM in toxin-binding sites were incubated with the indicated concentration of ligand for 20 min and 470 nM [125 I] α -BuTx was added to start the reaction. The initial rates, as determined from semilogarithmic plots of the data, were fit to a linearized form of the empirical relation $k_{\text{obs}} = k/(1 + L/K_i)$, in which k_{obs} was the initial rate in the presence of ligand concentration L , k the rate in the absence of ligand, and K_i the apparent inhibition constant. The lines were calculated using K_i values of 0.95 ± 0.06 mM for PAA (●) and 1.7 ± 0.1 mM for procaine amide (○). (B) Effects of PAA on the fluorescence of membrane bound ethidium: The concentrations of α -BuTx binding sites and of ethidium were 1 μ M. $\Delta F_{\text{corrected}}$ is the change in ethidium fluorescence for membrane fragments alone minus the corresponding change for membrane fragments that were incubated with an excess of α -BuTx.

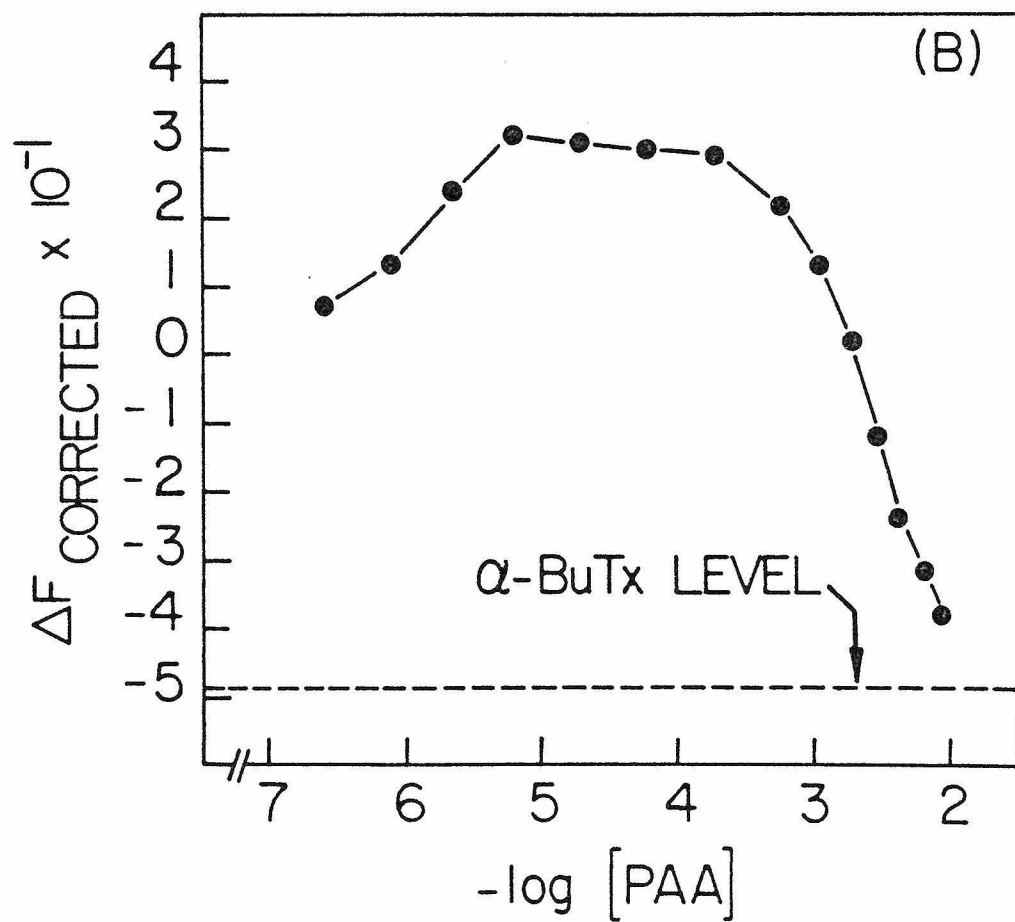
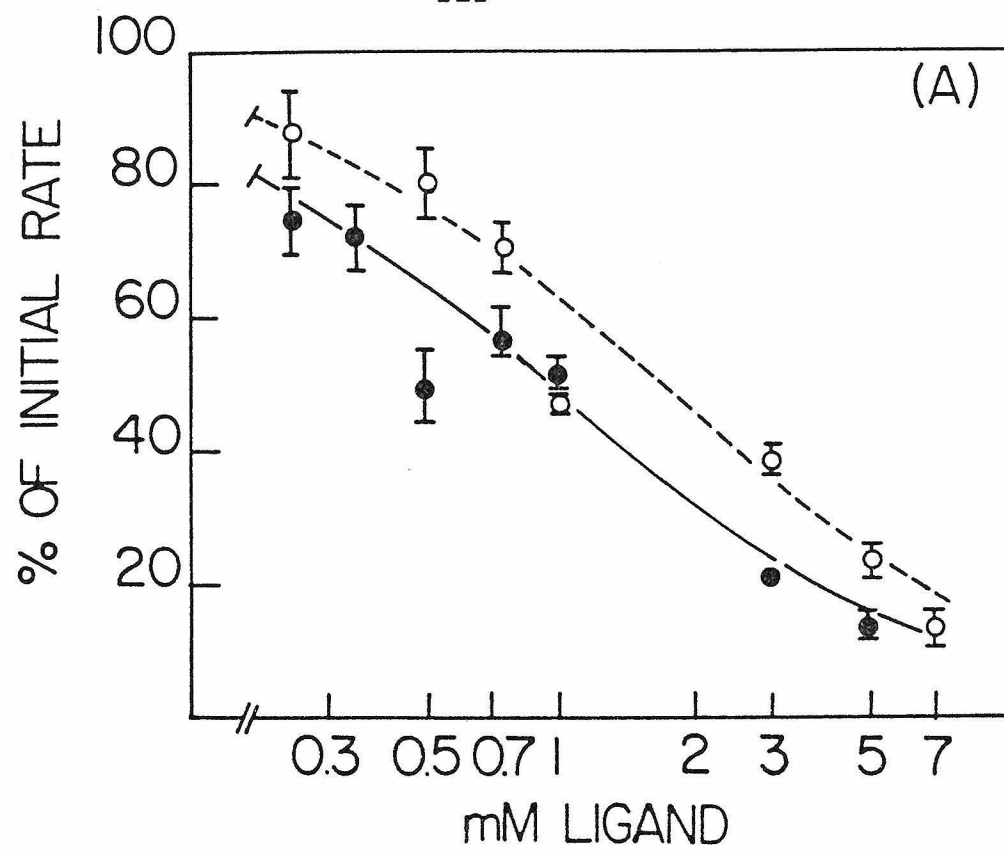
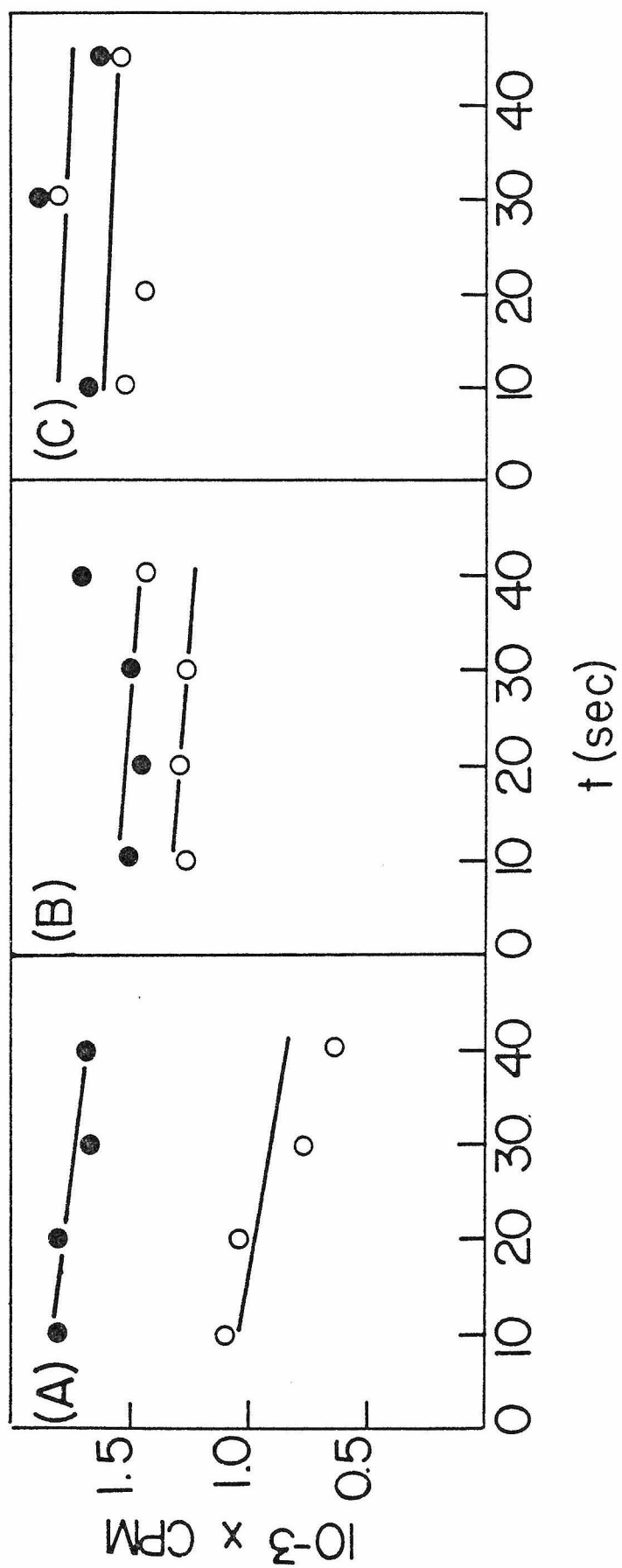


FIGURE 3: Inhibition of carbamylcholine-induced efflux of $^{22}\text{Na}^+$ by PAA: Membrane fragments which had been equilibrated with $^{22}\text{Na}^+$ were diluted 20-fold into buffer containing (A) 0 μM , (B) 50 μM , or (C) 500 μM PAA. After a 5 min incubation 100 μM carb (final concentration, $\bigcirc$) or buffer ($\bullet$) was added and the efflux of $^{22}\text{Na}^+$ was measured by the filtration assay of Miller et al. (1979). The final concentration of α -BuTx binding sites was $\sim 1.3 \mu\text{M}$.



Briley and Changeux, 1978; Blanchard et al., 1979; see also Chapter 2) the effects of PAA on the rate of this process were examined. Up to concentrations of 50 μ M PAA, the rate of the transition of the receptor from low to high affinity caused by 1 μ M carb (Lee et al., 1977) was only slightly changed. The half-time of 97 ± 23 sec for this process in the presence of 1 μ M carb alone was reduced to 71 ± 16 sec at the highest concentration of PAA tested, a 1.4 ± 0.4 -fold decrease.

(2) Labeling of Purified Receptor

The pattern of radioactivity observed after SDS-polyacrylamide gel electrophoresis of solubilized receptor (purified by affinity chromatography) that was labeled by [3 H] PAA is shown in Fig. 4. All four receptor bands were found to be labeled. Labeling occurred only on photolysis of [3 H] PAA and could be inhibited by addition of unlabeled PAA. No further studies of PAA labeling of solubilized AcChR were conducted and Fig. 4 is shown only for comparison to the patterns observed for AcChR-enriched membrane fragments.

(3) Pattern of Labeling of AcChR-Enriched Membrane Fragments

Figure 5A shows a SDS gel electrophoresis profile of membrane fragments that were photolabeled with [3 H] PAA. Only two labeled bands were seen. Although the relative mobility of the faster migrating band corresponded to a molecular weight of about 42,000-44,000 daltons, the limited resolution of the gels in this region made it impossible to determine if the 40,000 dalton receptor band also contained bound label. The amount of radioactivity incorporated in the

FIGURE 4: Profile of radioactivity incorporated into solubilized, purified AcChR on photolysis of [^3H]PAA: Concentrations were $0.7\ \mu\text{M}$ in [^3H]PAA. A total of $1.4\ \text{nmole}$ of α -BuTx binding sites was placed on the gel.

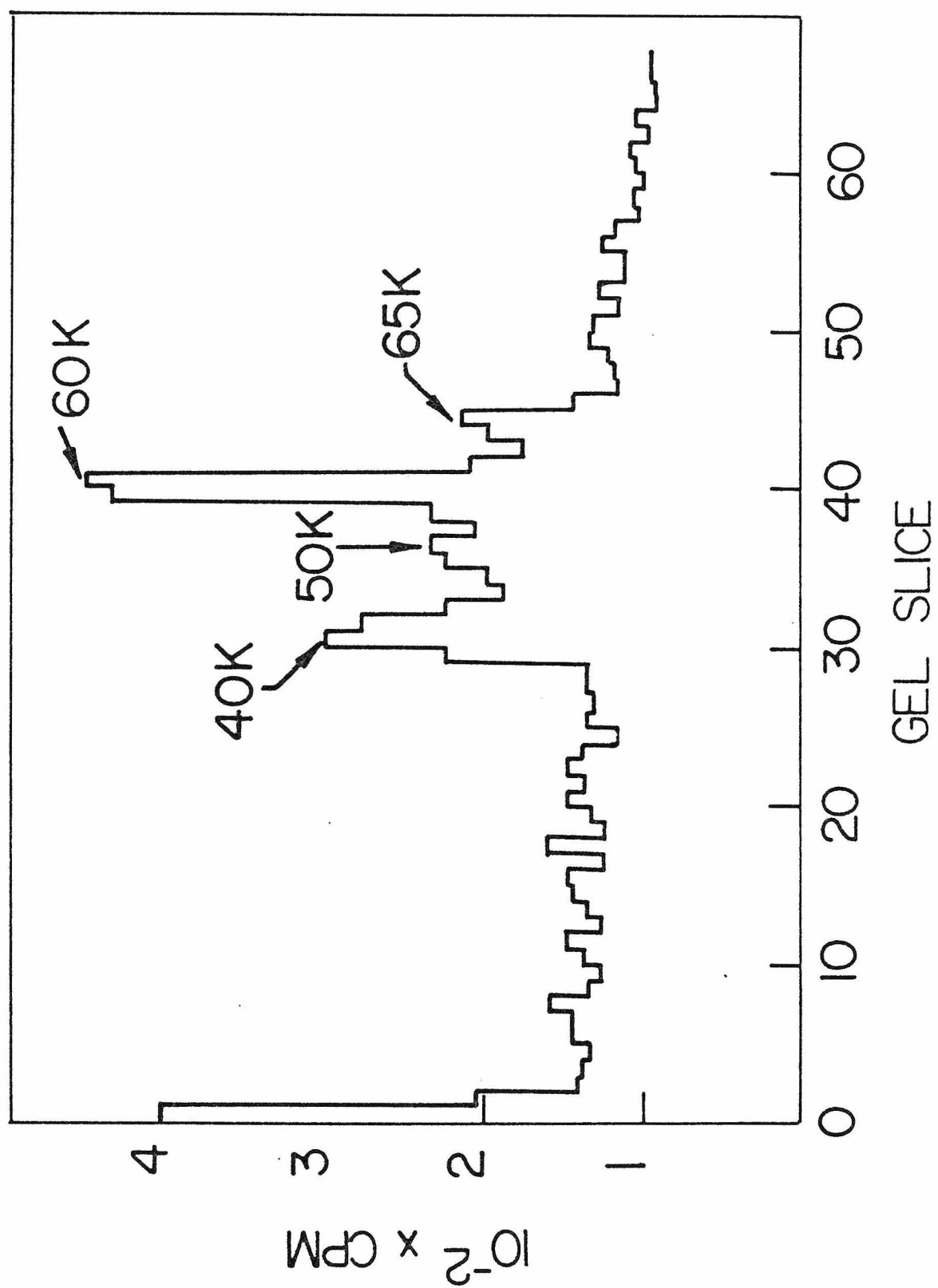
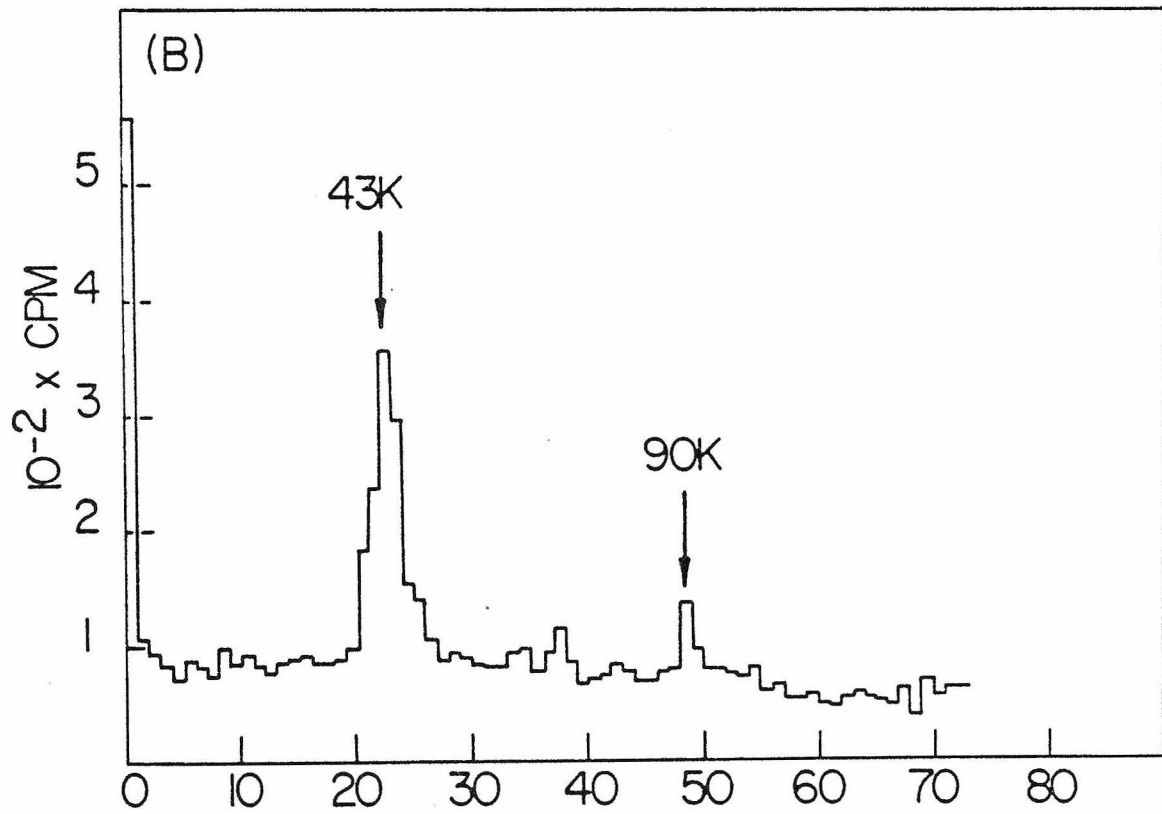
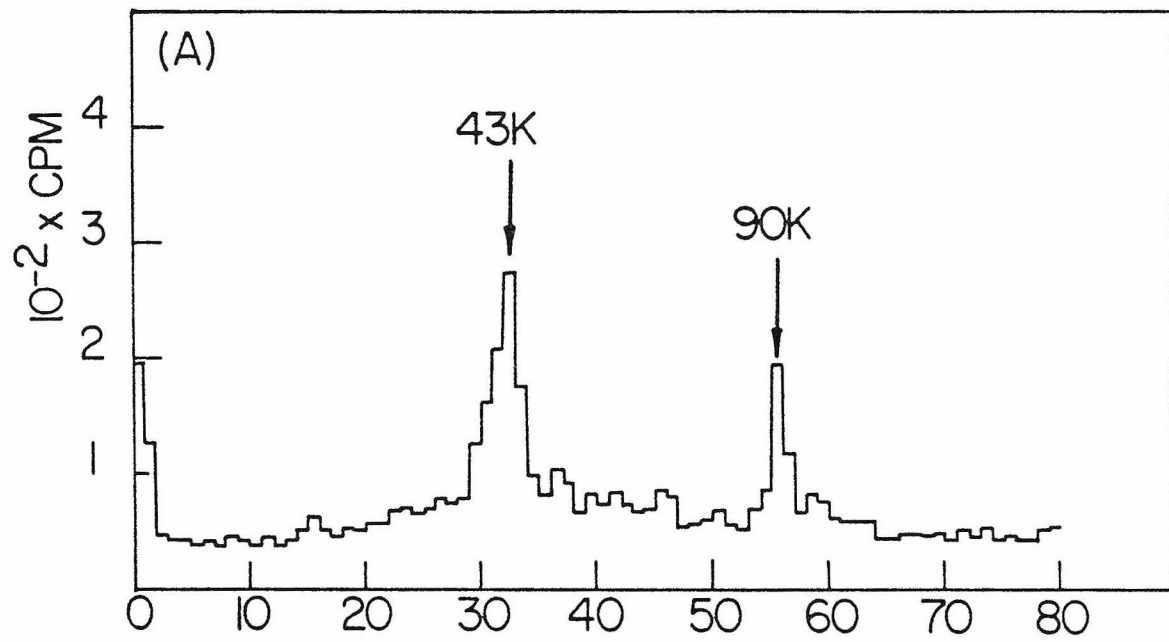


FIGURE 5: Polyacrylamide gel electrophoresis profiles of AcChR-enriched membrane fragments labeled with [^3H]PAA: (A) A solution 0.5 μM in [^{125}I] α -BuTx binding sites and 5 μM in [^3H]PAA was treated as described in Materials and Methods. A total of 0.75 nmol of [^{125}I] α -BuTx binding sites was placed on the gel. (B) Conditions were the same as A except that the electric tissue was homogenized in the presence of IACNH_2 . One nanomole of [^{125}I] α -BuTx sites was placed on the gel. Gel slice 1 is the position of the tracking dye; the last slice is the top of the gel.

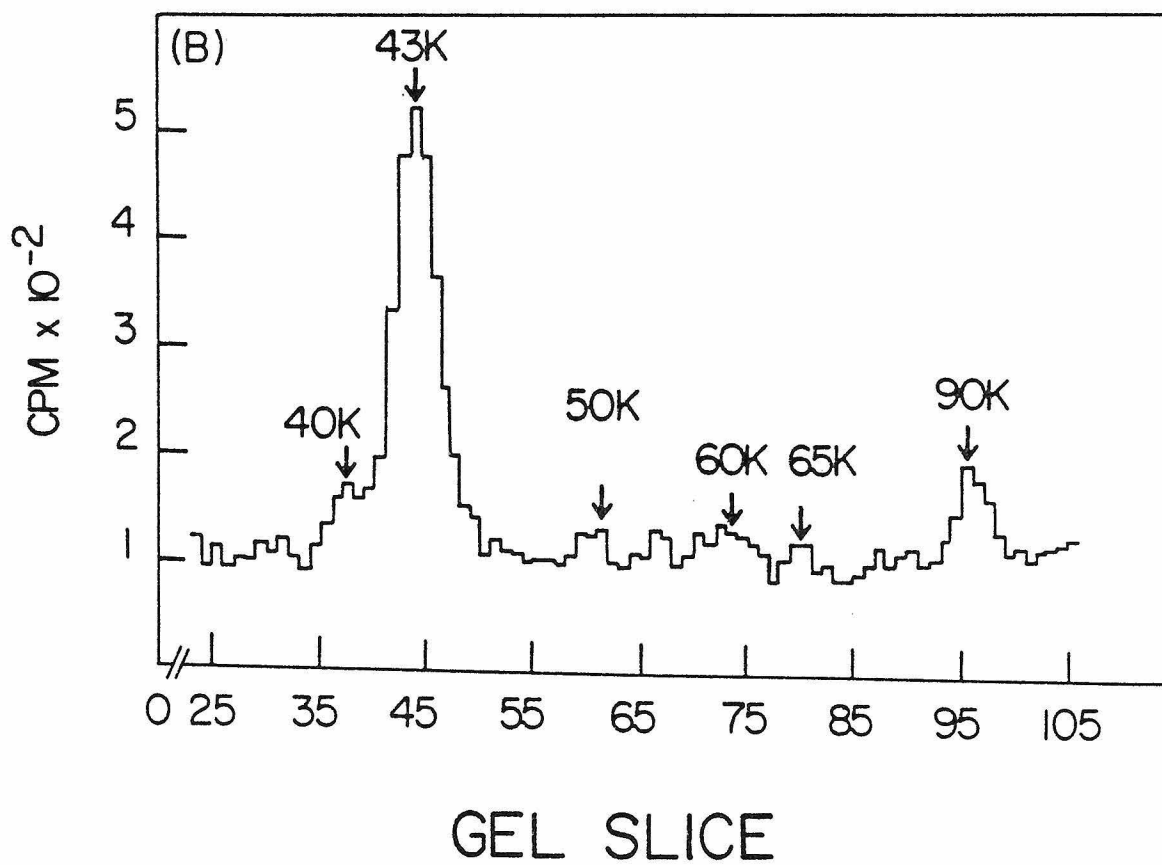
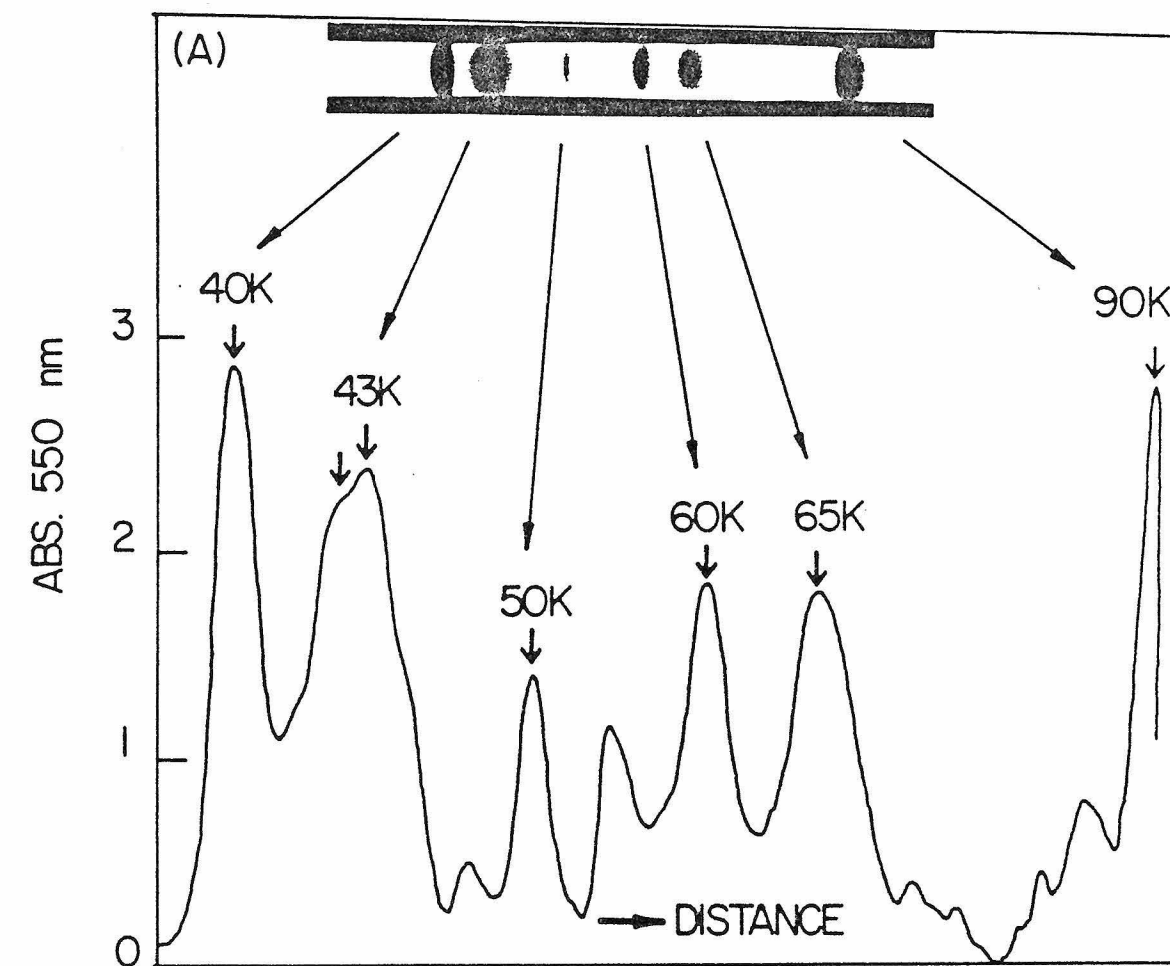


GEL SLICE

90,000 dalton band varied from preparation to preparation, as did the intensity of its Coomassie Brilliant Blue staining. When membrane fragments homogenized in the presence of IAcNH₂ were labeled under the same conditions, almost no radioactivity was incorporated into the 90,000 dalton band (Fig. 5B). The decrease in the intensity of the Coomassie Brilliant Blue staining was less dramatic. Two possibilities could explain the changed labeling pattern in IAcNH₂-treated membrane fragments: (i) the component of molecular weight 90,000 that was labeled by PAA was lost during the purification process, presumably because artifactual disulfide bonds could not form or (ii) IAcNH₂ altered the binding properties of the membrane fragments for PAA and this change was reflected in the labeling of the 90,000 dalton band. Because this band was labeled when membranes were treated with IAcNH₂ after preparation (not shown), the second possibility could be ruled out. In accord with the conclusion that IAcNH₂ treatment did not perturb the interactions of PAA with membrane fragments, the ethidium fluorescence signal observed when membranes were titrated with PAA, was unchanged. Both the increased fluorescence at low concentrations and the decrease in signal at higher ones were observed.

When membranes were run on gels 20 cm long, the separation between the 40,000 and 43,000 dalton bands was increased. As Fig. 6 shows, both bands were labeled, with the majority of the radioactivity in the band of higher molecular weight. Photolysis of membrane fragment solutions containing both [³H]PAA and 2-5 μM carb resulted in decreased incorporation of radioactivity into the 40,000 dalton but not the 43,000 dalton band (or the residual 90,000 dalton label, not

FIGURE 6: Improved separation of membrane components on 1×20 cm SDS/polyacrylamide gels: (A) Scan of Coomassie Brilliant Blue staining intensity. The central portion of a 1×20 cm gel was scanned with a Gilford linear transport accessory with a specially made cuvette and holder. Note that the region around 43,000 daltons contained several staining bands that were not well resolved. The relative amounts of each polypeptide could not be quantitated due to the high absorbance and the fact that the stain did not always penetrate to the center of the gels because of their large diameter. (B) radioactivity profile. A total of 2 nmol of [^{122}I] α -BuTx sites was placed on the gel. The labeling conditions were identical to those in Fig. 5B. The major amount of radioactivity was incorporated at a position corresponding to a molecular weight of 43,000 with minor bands at 40,000 and 90,000 daltons. Each gel slice was 1.1 mm long, with a total of 152 slices from the dye front to the top of the gel.



shown). In addition, photolysis in the presence of micromolar concentrations of HTX did not affect the labeling pattern.

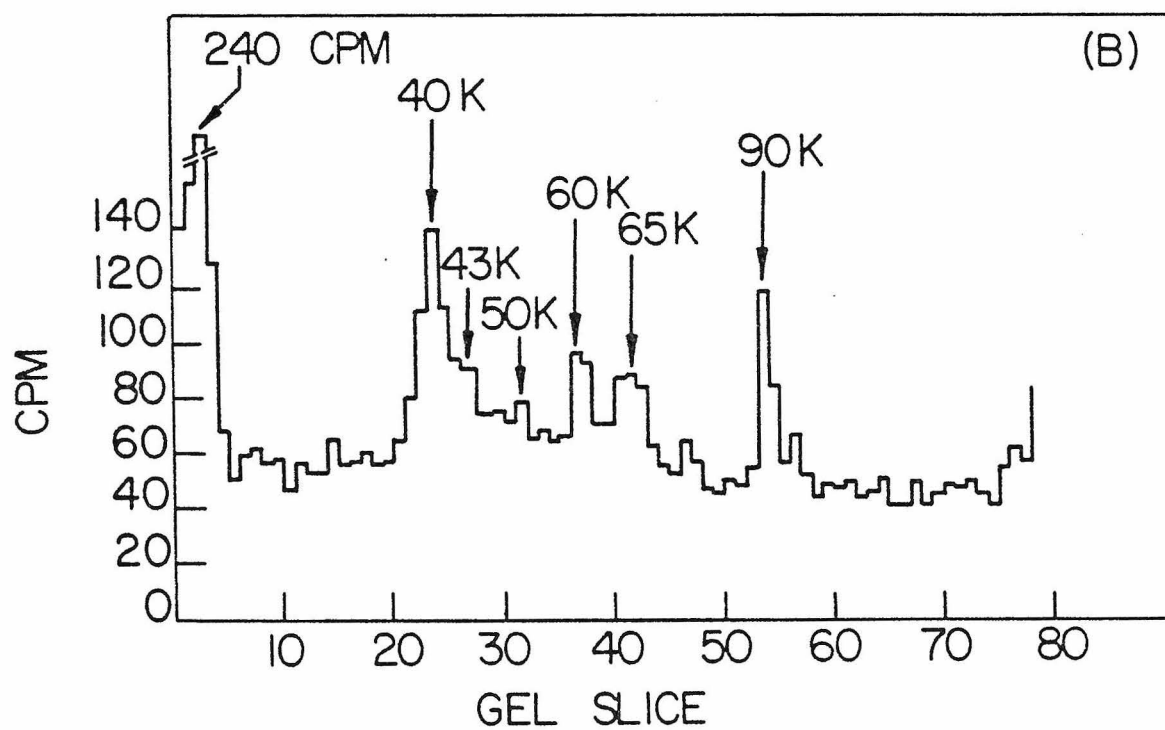
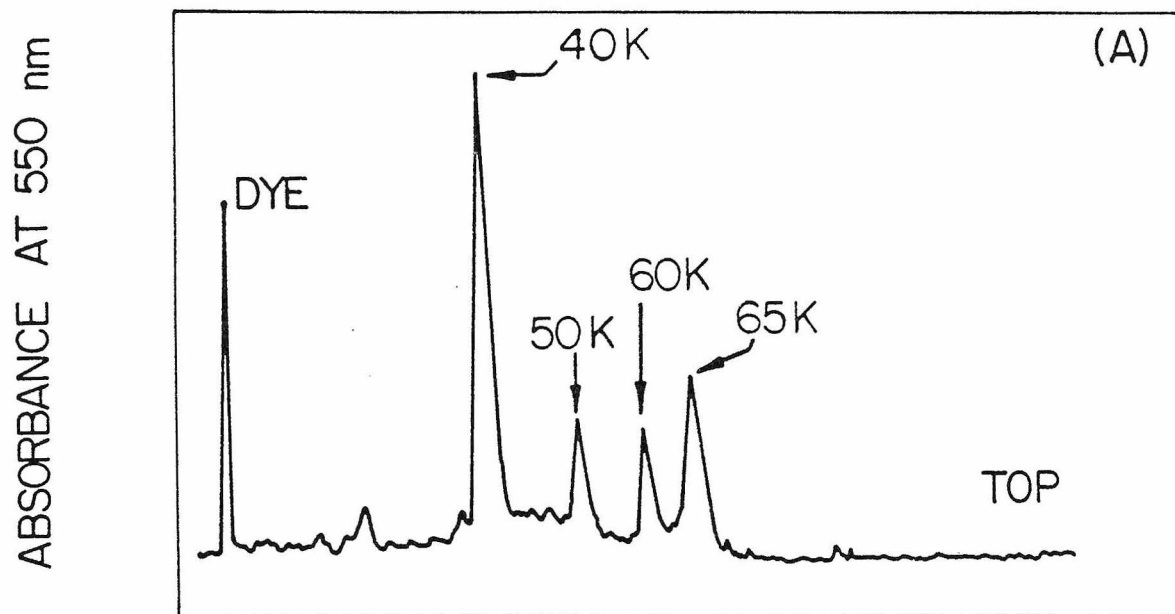
The 40,000 dalton subunit of T. californica AcChR contains sites for binding of α -toxins (Hucho et al., 1976; Witzemann and Raftery, 1978; Witzemann et al., 1979) and cholinergic ligands such as the antagonists MBTA (Weill et al., 1974) and DAP-azide (Witzemann and Raftery, 1977) and the agonist bromoacetylcholine (Moore and Raftery, 1979). The label incorporated into the polypeptide of 40,000 daltons was therefore attributed to PAA binding at these site(s). The small amount of bound radioactivity was probably a reflection of the low affinity (determined by the inhibition of [125 I]- α -BuTx binding) of these site(s) for the anesthetic analog.

(4) Labeling of Base-Treated AcChR Membrane Fragments

Treatment of AcChR-enriched membrane fragments with base at pH 11 results in a preparation which contains essentially only the four receptor polypeptides (see Chapter 1). Such preparations have recently been used to demonstrate that the Coomassie Brilliant Blue staining bands of $\sim 43,000$ and $\sim 90,000$ daltons are not required for ion translocation (Moore et al., 1979), or for the binding of HTX (Elliott et al., 1979) or an HTX competitive local anesthetic analog (Neubig et al., 1979). Although these results suggested that the 43,000 dalton band labeled by [3 H] PAA was not necessary for AcChR function it was also possible that [3 H] PAA labeled a 43,000 dalton component (other than that component(s) removed by base treatment) which was not stained by Coomassie Brilliant Blue. Therefore, the labeling of base-treated

AcChR membrane fragments by [^3H]PAA was examined. Figure 7 shows that the pattern of radioactivity obtained for base-treated membranes was more complicated than that seen for untreated preparations although the total amount of incorporated label was much smaller. The results indicated that base treatment removed the component of 43,000 daltons that was labeled by [^3H]PAA. Labeling of the 40,000 dalton component and the residual 90,000 dalton component was still observed in the base-treated membranes. In addition, the 60,000 and 65,000 dalton bands were also labeled by [^3H]PAA. The low levels of radioactivity that were incorporated (compare, e.g., Figs. 5B and 6B) suggested that the 60,000 and 65,000 dalton subunits were also labeled in untreated membrane preparations but that this labeling was obscured by a higher background in the membranes of lower specific activity. The very low levels of label incorporated in the 60,000 and 65,000 subunits ruled out further study of the specificity of the binding of [^3H]PAA to these bands.

FIGURE 7: Labeling of Base-treated AcChR-membrane fragments by [^3H]-PAA: (A) Coomassie Brilliant Blue staining intensity of a sample of the base-treated membranes labeled below. (B) Solutions containing $5\ \mu\text{M}$ [^3H]PAA and $0.5\ \mu\text{M}$ α -BuTx binding site were irradiated as described in Materials and Methods and a total of 2 nmol of α -BuTx sites was run on the gel.



Discussion

The postsynaptic membrane responds to cholinergic agonists by a transient increase in cation permeability, and this response is directly inhibited by compounds that block the binding of agonists. Indirect inhibition may occur by conversion of the AcChR-ion translocation complex to an inactive conformation (desensitization) or by perturbation of the structure(s) involved in ion translocation or of its coupling to the AcCh binding site. Tertiary amine local anesthetics affect the rate of both the in vivo process of desensitization (Magazanik and Vyskocil, 1973; Magazanik, 1976) and of its in vitro correlate (Weiland et al., 1977; Briley and Changeux, 1978; Chapter 2). Local anesthetics also interfere with the mechanism of ion translocation (Furakawa, 1957; Maeno, 1966; Steinbach, 1968a, 1968b; Ruff, 1976; Neher and Sakmann, 1976; Steinbach, 1977).

PAA inhibited the carb-induced efflux of $^{22}\text{Na}^+$ as was expected for a local anesthetic. The effects of PAA on the rate of the conversion of the membrane-bound receptor from a state of low to one of high affinity by carb further confirmed that this compound acted as a local anesthetic in vitro. PAA decreased the half-time of this transition by a factor of only 1.4 ± 0.4 at a concentration 10 times above that used in the labeling experiments. This value was in agreement with the observation that some local anesthetics (e.g., procaine and lidocaine) have little effect on the rate of this transition in receptor affinity (see Chapter 2). Little or no effect on this rate at a given concentration of local anesthetic could indicate either (a) that little or no local anesthetic

was bound to the site affecting the rate of conversion, or (b) that the local anesthetic was bound to this site but did not affect this rate to an appreciable extent. Compounds such as some local anesthetics (Weiland et al., 1977; Briley and Changeux, 1978), detergents (Briley and Changeux, 1978; Brisson et al., 1975; Elliott, 1979), caeruleo-toxin (Bon and Changeux, 1975; Moody and Raftery, 1978), and, in some tissues (Burgermeister et al., 1977) but not others (Elliott and Raftery, 1977), HTX affect the rate of transition of receptor from a state of low to one of high affinity. However, it is not clear if these diverse compounds modify the rate of the transition in receptor affinity by a common mechanism.

The amount of radioactivity incorporated into the protein of 90,000 daltons varied from one preparation to another. The possibility that this band was a complex of smaller polypeptides crosslinked by disulfide bonds was ruled out because dithiothreitol was included in the sample buffer for all SDS/polyacrylamide gel electrophoresis experiments. The band of molecular weight 90,000 daltons was tentatively assumed to be unrelated to the AcChR-ion-translocation complex since it was easily separable from the membrane fragments on homogenization in the presence of IAcNH₂.

Recently, base-treated membranes which lack the 90,000 and 43,000 dalton components have been used to demonstrate that these polypeptides are not required for normal AcChR function. Specifically, base-treated membrane preparations (a) release vesicle entrapped ²²Na⁺ on addition of agonists (Moore et al., 1979), (b) bind HTX (Elliott et al., 1979) and an HTX competitive local anesthetic analog (Neubig et al.,

1979), and (c) exhibit in vitro desensitization (Elliott et al., 1980; see also Chapter 1) and the rate of this process is modulated by local anesthetics (Changeux et al., 1979; J. Miller and M. A. Raftery, unpublished). Therefore, neither the 43,000 or 90,000 dalton polypeptide is required for AcChR function although a structural role for one or both of these proteins cannot be ruled out.

Local anesthetics are defined as compounds that cause a reversible block of nerve conduction (Ritchie and Greengard, 1966). These compounds block axonal transmission by interacting with the voltage sensitive sodium channel or nerve (Strichartz, 1973; Courtney, 1975; Hille, 1977; Cahalan, 1978; Cahalan and Almers, 1979). In addition to their action on axonal transmission and the AcChR, local anesthetics are known to affect a wide variety of biological systems. Local anesthetics inhibit the catalytic activity of a number of enzymes including acetylcholinesterase (Skou, 1956), phospholipase A₂ (Scherphof and Westenberg, 1975; Kunze et al., 1976; Hendrickson and van Dam-Mieras, 1976), and firefly luciferase (Ueda et al., 1976). These compounds also affect the distribution and mobility of cell surface receptors (Poste et al., 1975a,b; Nicolson and Poste, 1976) and inhibit anion transport in erythrocytes (Gunn and Cooper, 1975; Low, 1978). The displacement of calcium ions may be involved in the local anesthetic inhibition of many such processes (Low et al., 1979). Because local anesthetics interact with such a wide variety of biological molecules, it is not surprising that PAA labeled other polypeptides in addition to the AcChR in Torpedo californica membrane fragments.

Since incorporation of [^3H]PAA into the protein of molecular weight 40,000 was prevented by the presence of carb, the labeling seen on this polypeptide could be interpreted as being due to PAA bound at cholinergic ligand binding sites. The addition of micromolar concentrations of HTX did not decrease the amount of radioactivity incorporated (upon photolysis of [^3H]PAA) in membrane fragments that had not been treated with base. Because there was little radioactivity incorporated in the 60,000 and 65,000 dalton polypeptides it is possible that HTX protected one or both of these bands from labeling by [^3H]PAA and that this protective effect could not be observed by the method used. Another possibility is that HTX and PAA bind to different sites. This idea is supported by the finding that PAA displaced [^3H]- H_{12} -HTX with an apparent K_I of 500 μM (J. Elliott, personal communication), a concentration 2 orders of magnitude higher than that used in the labeling experiments, and, as shown in Fig. 3, well above the concentrations of PAA needed to inhibit carb-mediated $^{22}\text{Na}^+$ efflux.

The labeling of T. californica acetylcholine receptor membrane fragments by [^3H]PAA was specific for polypeptide components of 43,000 and 90,000 daltons as well as three of the four AcChR polypeptides (i.e., the 40,000, 60,000 and 65,000 dalton bands). Because only a few of the many proteins present in the AcChR enriched membranes were labeled by [^3H]PAA and this labeling was inhibited by unlabeled PAA the incorporation of radioactivity into the bands of 40,000, 43,000, 60,000, 65,000 and 90,000 daltons was likely to result from [^3H] PAA bound to these proteins and not from free nitrene in the aqueous phase. Levy et al. (1977) obtained a similar result in their

studies of the labeling of hepatocyte plasma membranes by procaine azide. In this complex system only a single polypeptide of 21,400 daltons was labeled, even at millimolar concentrations of procaine azide. The present studies have indicated that photolabile local anesthetic analogs must be used with caution in the investigation of local anesthetic binding sites since labeling may also occur at sites of local anesthetic interaction unrelated to the protein that one wishes to study.

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CHAPTER 4: Kinetics of Carbamylcholine Binding to Membrane-Bound Acetylcholine Receptor Monitored by Fluorescence Changes of a Covalently Bound Probe.

Introduction

Acetylcholine receptor (AcChR) enriched membrane fragments prepared from Torpedo electric organs have been extensively used for in vitro studies of the ligand binding and functional properties of the nicotinic receptor in its membrane environment.

The kinetics of ligand binding to AcChR preparations have been studied by rapid-mixing experiments in which various fluorescence techniques have been used to monitor complex formation and consequent conformational changes. The effect of AcCh binding on the intrinsic fluorescence of membrane bound AcChR has been described (Bonner et al., 1976; Barrantes, 1976). Although this is the most direct method it lacks sensitivity due to the weak fluorescence of the receptor protein and the small signal change on ligand binding. Recently there have been reports of the kinetics of binding of fluorescent analogues of acetylcholine (Jürss et al., 1979; Heidmann and Changeux, 1979). These analogues have been shown to be agonists at the neuromuscular junction but this method suffers from the disadvantage that ambiguity in the interpretation of the kinetic signals may arise from extrapolation of the effects of these compounds to describe the behavior of well characterized agonists such as acetylcholine and Carb. Extrinsic fluorescence probes such as quinacrine (Grünhagen and Changeux, 1976; Grünhagen et al., 1976, 1977) and ethidium (Schimerlik et al., 1979; Quast et al., 1978, 1979) have also been used to monitor the interaction of agonists and AcChR-enriched membrane fragments. However, quinacrine acts as a local anesthetic

(Grünhagen and Changeux, 1976) and thus itself affects the binding of agonists. In addition the possibility of uptake and dissociation of non-covalently bound probes during the course of an experiment as a result of ligand induced conformational changes cannot be discounted (Schimerlik et al., 1979).

This chapter describes an alternative method for monitoring agonist binding to membrane bound AcChR from Torpedo californica. This approach makes use of ligand binding induced fluorescence changes of a probe covalently bound to the receptor. Nicotinic AcChR's are known to contain a disulphide bond in the vicinity of an agonist or antagonist binding site (Karlin, 1969) which, after reduction, can be readily alkylated by affinity reagents such as MBTA (Weill et al., 1974) and the agonist 2-bromoacetylcholine (Chang et al., 1977; Damle et al., 1978; Moore and Raftery, 1979a). This disulphide is therefore a convenient target for attack by a sulphydryl selective alkylating reagent. The fluorescent probe we have used for this purpose is 5-iodoacetamido salicylic acid (IAS) which has previously been used in fluorescence energy transfer studies of rhodopsin (Wu and Stryer, 1972).

We have measured the equilibrium binding properties of the specifically modified receptor and also the rapid kinetics of Carb binding. The results obtained are consistent with a more generalized form of a model previously proposed for agonist binding by Quast et al., (1978, 1979).

Materials and Methods

(1) Materials

IAS was purchased from Molecular Probes, Inc., [^3H] bromoacetylcholine was a generous gift from H.-P. Moore. The sources of all other materials are described in the previous chapters.

(2) Covalent Labeling of AcChR Membrane Fragments with IAS

Covalent modification of AcChR-enriched membrane fragments by IAS was carried out as follows. Membrane fragments were diluted to a concentration of 1.0-1.5 μM in α -BuTx binding sites in Ca^{2+} free Hepes Ringers (20 mM Hepes, 250 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , 0.02% NaN_3 , pH 7.4) which had been flushed with argon. DTT was added to a final concentration of 50 μM and reduction was allowed to proceed for 1 hour at 4°. IAS was added from a stock concentration of 25 mM in 200 mM Tris, pH 8.5 to give a final concentration of 150-250 μM . The reaction mixture was shielded from light and, after adjustment of its pH to 7.5, was stirred for 2 hours at 4°. The mixture was then diluted with the same buffer and centrifuged at 30,000 rpm for 45 min in a Beckman Type 35 rotor. The pellet was resuspended in a large volume of buffer and centrifuged as before. Final resuspension was in Ca^{2+} free Ringers to give a final concentration of approximately 10 μM in α -BuTx binding sites. Covalent and reversible binding of bromoacetylcholine were carried out using the procedures of Moore and Raftery (1979a).

(3) Fluorescence Experiments

All equilibrium and kinetic experiments were carried out using Hepes Ringers containing 4 mM CaCl_2 . Fluorescence spectra were recorded on a Perkin-Elmer MPF-4 spectrofluorimeter thermostatted at $25^\circ \pm 1^\circ$. The equilibrium dissociation constant for the Carb-IAS labeled receptor complex was obtained from a fluorescence titration using excitation and emission wavelengths of 282 ± 4 nm and 430 ± 4 nm respectively. 5 μl aliquots of Carb solution were added to 3 ml of membrane fragments in a 1 cm^2 cuvette. Equilibrium fluorescence measurements were recorded 5 min after ligand addition. In order to minimize photolytic reactions samples were shielded from the light source between measurements. Small corrections for nonspecific effects were made using the results of a parallel titration of IAS-labeled AcChR which had been pre-equilibrated with α -BuTx. Dissociation constants were calculated by a non-linear regression method as described in Chapter 2. Fluorescence titration data were fitted to the appropriate equations derived for the mechanism considered. Rapid mixing experiments were carried out as described in Chapter 2 except that the excitation wavelength was 290 nm and fluorescence emission was monitored using a Corning 0-52 filter (50% transmission at 360 nm).

Results

(1) Fluorescence Properties of IAS Modified Membrane Fragments:

The fluorescence excitation spectrum of IAS-labeled membrane fragments is shown in Fig. 1. Two peaks of fluorescence intensity were observed corresponding to excitation of the bound IAS either directly (~ 320 nm) or via energy transfer from the AcChR (~ 290 nm). When IAS-labeled membrane fragments were excited at 320 nm, the excitation maximum of free IAS, maximum fluorescence emission was observed at 430 nm as shown in Fig. 2C. Similar fluorescence emission was found when excitation was via energy transfer from the protein at 282 nm although this spectrum was complicated by the light scattering of the membrane fragments (Fig. 2B). Addition of $100\ \mu\text{M}$ Carb led to an enhancement of the fluorescence of bound IAS showing that the probe was sensitive to agonist binding. The observed increase in fluorescence was greater when excitation was at 282 nm suggesting that the probe was indeed associated with the protein component of the membrane-bound receptor (Fig. 2).

Figure 2A shows the fluorescence spectrum of membrane fragments which were not reduced with DTT prior to reaction with IAS. Considerable non-specific labeling is apparent indicating the existence of free sulphydryl groups in the membranes even though they had been prepared in the presence of excess IAcNH_2 (Elliott *et al.*, 1980; Chapter 1). These groups were not significantly protected from IAS modification by further treatment with iodoacetamide, a result which may be due to differing reactivities of the two alkylating agents. However, membrane

FIGURE 1: Fluorescence Excitation Spectrum of IAS-Labeled Membrane Fragments: The emission wavelength was 430 nm and the concentration of α -BuTx binding sites was 0.5 μ M.

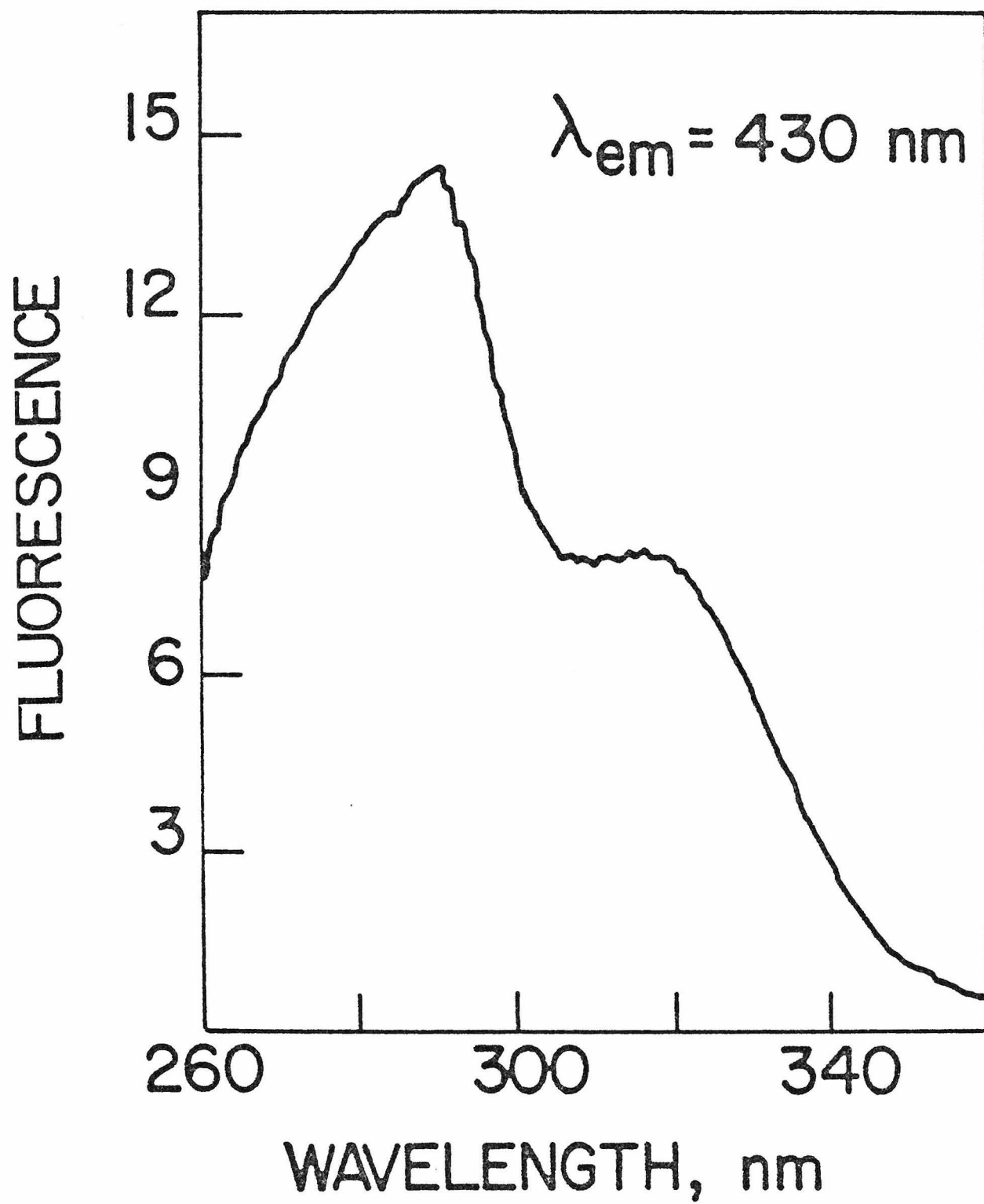
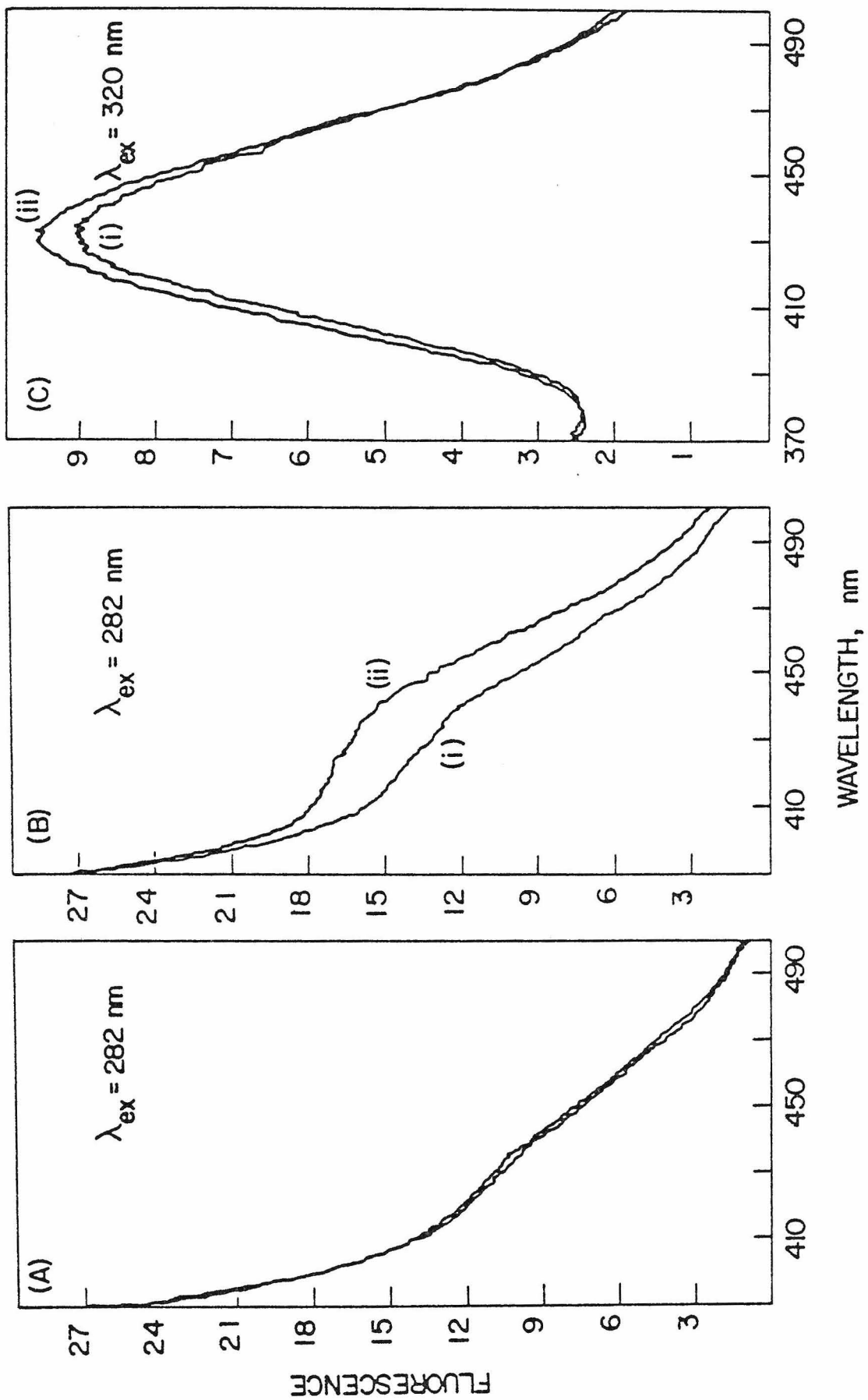


FIGURE 2: Fluorescence Emission Spectra of IAS-Labeled Membrane Fragments: The AcChR concentration was $0.5\ \mu\text{M}$ in α -BuTx binding sites and spectra were recorded either (i) in the absence of or (ii) in the presence of $10\ \mu\text{M}$ Carb.

(A) Spectra of AcChR-enriched membrane fragments which were reacted with $150\ \mu\text{M}$ IAS without prior reduction of the AcChR by DTT.

(B) Energy transfer and (C) direct excitation spectra of labeled membrane fragments which were prepared as described in the text by reduction with $50\ \mu\text{M}$ DTT followed by alkylation with $150\ \mu\text{M}$ IAS.



fragments which had been labeled without DTT reduction did not show a fluorescence enhancement on addition of Carb. It can therefore be concluded that the Carb induced fluorescence change was not due to IAS modification of free sulphydryl groups present in the non-reduced membrane preparations.

(2) Extent of IAS Labeling:

It has been shown that after reduction of the AcChR molecule by DTT it is readily alkylated by the agonist bromoacetylcholine (Moore and Raftery, 1979a). It seems likely in view of the relatively large signal change occurring on Carb binding to the IAS-modified receptor that the disulphide which is specifically modified by IAS is also in the vicinity of the agonist binding site. An attempt to assess the extent of labeling of this disulphide has therefore been made assuming competitiveness between IAS and bromoacetylcholine. Following inhibition of the acetylcholinesterase activity by addition of diethylnitrophenyl phosphate to the membrane preparations, control and IAS-membrane fragments were reduced with 40 μ M DTT prior to reaction with excess [3 H] bromoacetylcholine as described by Moore and Raftery (1979a). The extent of IAS labeling was estimated from the difference in bromoacetylcholine covalently bound to modified and control membrane preparations. For this particular preparation, where 150 μ M IAS was used in the modification procedure, the extent of labeling was 54% of the bromoacetylcholine sites. It is clear from recent results that an increased fluorescence change on Carb addition and thus a higher degree of labeling can be obtained by increasing the concentration of

IAS used in the treatment to 250 μ M and by more careful control of the DTT reduction step.

Reversible binding of bromoacetylcholine was measured by centrifugation assay. This experiment revealed that IAS treatment did not alter the ability of membrane bound AcChR to bind this agonist reversibly suggesting that IAS presence did not significantly affect the reversible binding of agonists.

(3) Effect of IAS Modification on the Ligand Binding Properties of the AcChR:

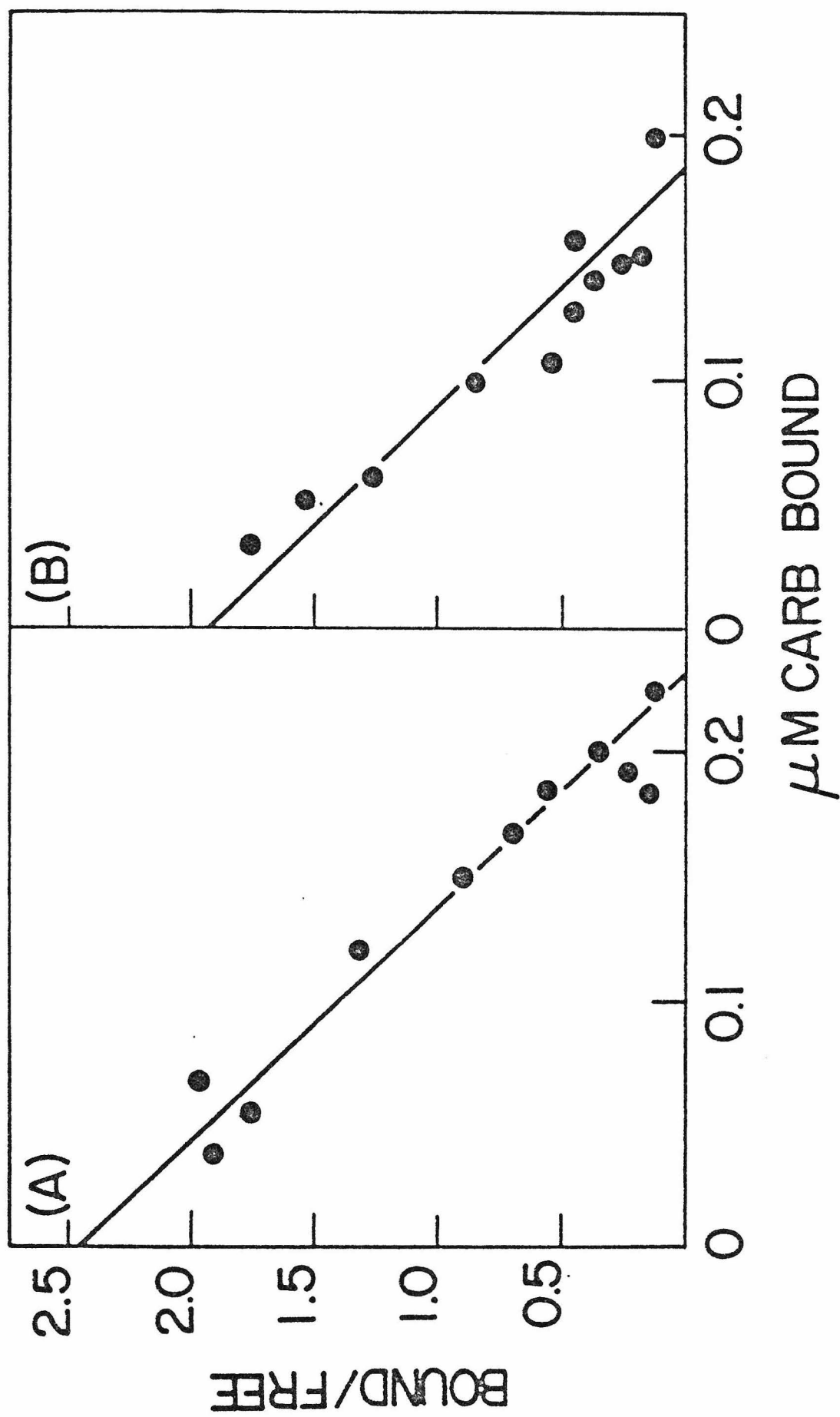
Measurement of α -BuTx binding activity and protein concentration of membrane fragments before and after treatment with IAS have shown that modification did not affect the specific activity of the membrane preparation. IAS-labeled membrane fragments retained the ability to undergo a change to a state of higher affinity for agonists on exposure to low concentrations of Carb.

The dissociation constant for Carb binding and number of binding sites were measured by a centrifugation assay using [3 H] Carb. The results shown in Fig. 3 suggest that IAS treatment did not alter either the K_d for Carb binding (approximately 100 nM) or the stoichiometry of Carb and α -BuTx binding sites. No evidence for heterogeneity of binding sites was found. These binding assays have been repeated using several different membrane preparations and similar results were obtained from each experiment. On a few occasions a small reduction in the apparent number of Carb sites (to about 75% of the control) was observed after treatment. This may be attributable to

FIGURE 3: Scatchard Plots of [^3H] Carb Binding to AcChR-Enriched Membrane Fragments:

(A) [^3H] Carb binding to control membrane fragments. The AcChR concentration was $0.25\ \mu\text{M}$ in α -BuTx sites. A linear least squares fit to the data gave values of $93 \pm 7\ \text{nM}$ for the K_d and $0.23 \pm 0.02\ \mu\text{M}$ for the concentration of high affinity Carb binding sites.

(B) [^3H] Carb binding to IAS labeled membrane fragments. AcChR concentration was the same as in (A) and data fitting gave values of $94 \pm 11\ \text{nM}$ for the K_d and $0.18 \pm 0.02\ \mu\text{M}$ for the concentration of Carb binding sites.



partial blocking of the receptor in a low affinity form for Carb as a result of reduction and alkylation as described by Moore and Raftery (1979b).

The results of a fluorescent titration of IAS labeled membrane fragments by Carb are shown in Fig. 4. A nonlinear regression fit to these data after correction for bound ligand gave a K_d value of 171 ± 34 nM. This is in very good agreement with the value obtained for the same preparation by centrifugation assay using radioactive ligand (shown in Fig. 3) and indicates that the fluorescence changes of bound IAS is a sensitive and accurate monitor of Carb binding.

(4) Qualitative Description of the Kinetics of Carb Binding to IAS-Labeled Membrane Fragments:

The reaction traces obtained when Carb was rapidly mixed with labeled receptor were complex and could be resolved into four distinct components. Fortunately these four phases were fairly well separated in rate which enabled kinetic parameters for each phase to be obtained by careful control of the timing used in data collection (Fig. 5).

The fastest phase (Phase 1, Fig. 5A) having an apparent rate of approximately 30 s^{-1} was sufficiently well separated temporally that it could be analyzed in isolation and was fitted to a single exponential equation. The three slower phases were monitored over several time courses by varying the sampling rate for data collection as shown in Fig. 5B-D. Most of these reaction traces were fitted to a sum of two exponentials. For further analysis and mechanism-fitting, parameters for any phase which had been obtained from traces in which the time

FIGURE 4: Fluorescence Titration of IAS-Labeled Membrane Fragments by Carb: Small volumes of Carb solution were added to 3 ml labeled receptor and the fluorescence enhancement (in arbitrary units) was measured using excitation and emission wavelengths of 282 nm and 430 nm, respectively. The concentration of Carb binding sites ($0.37 \mu\text{M}$) was estimated from the results of centrifugation assays using radioactive Carb (Fig. 2). The data were fitted to a simple binding equation, $F = F_0 \cdot L_f / (K_d + L_f)$ where F is the observed fluorescence, F_0 is the equilibrium fluorescence and L_f is the free ligand concentration. The solid line was calculated from the best fit parameters, $K_d = 171 \pm 34 \text{ nM}$ and $F_0 = 10.1 \pm 0.4$.

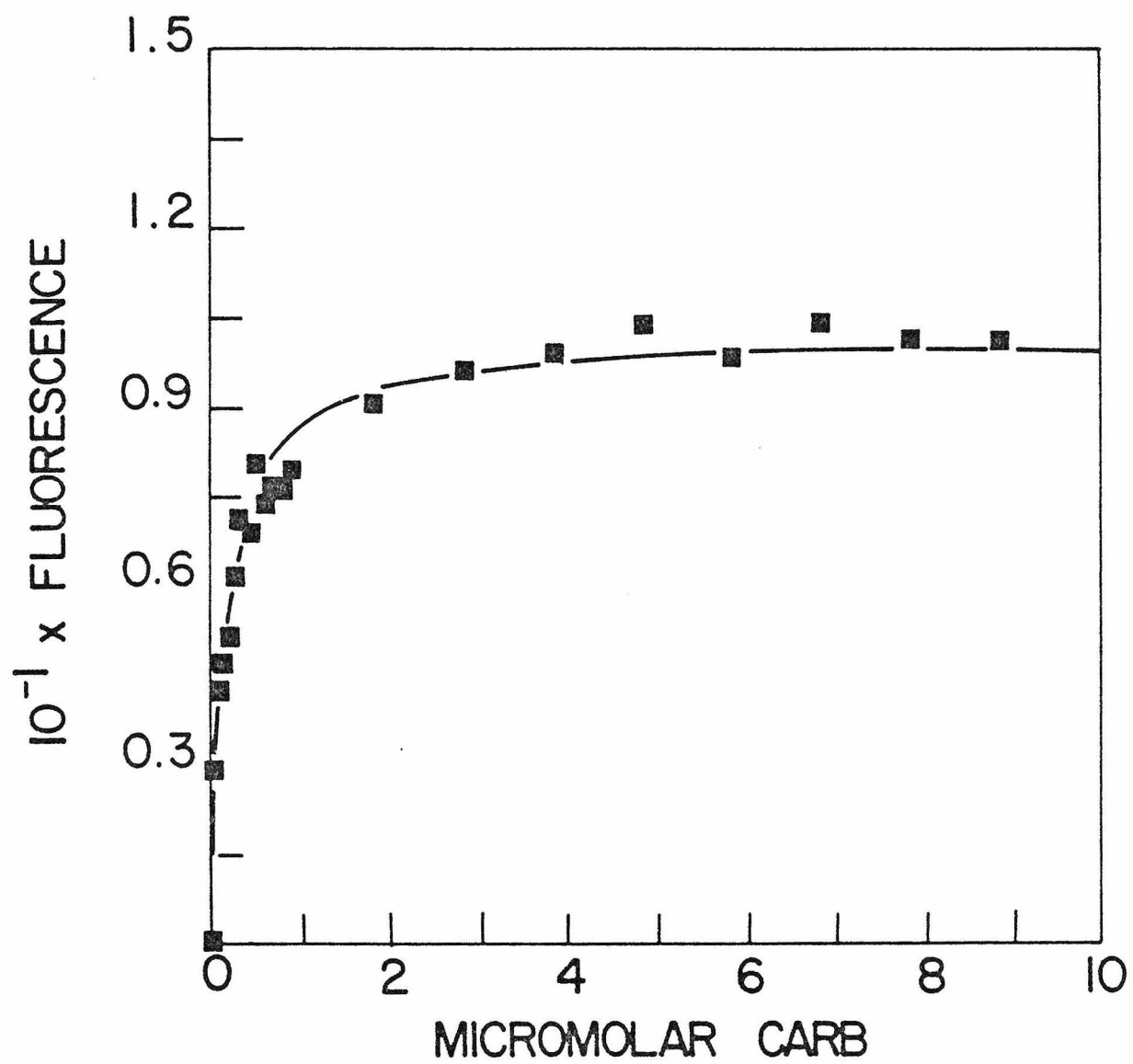


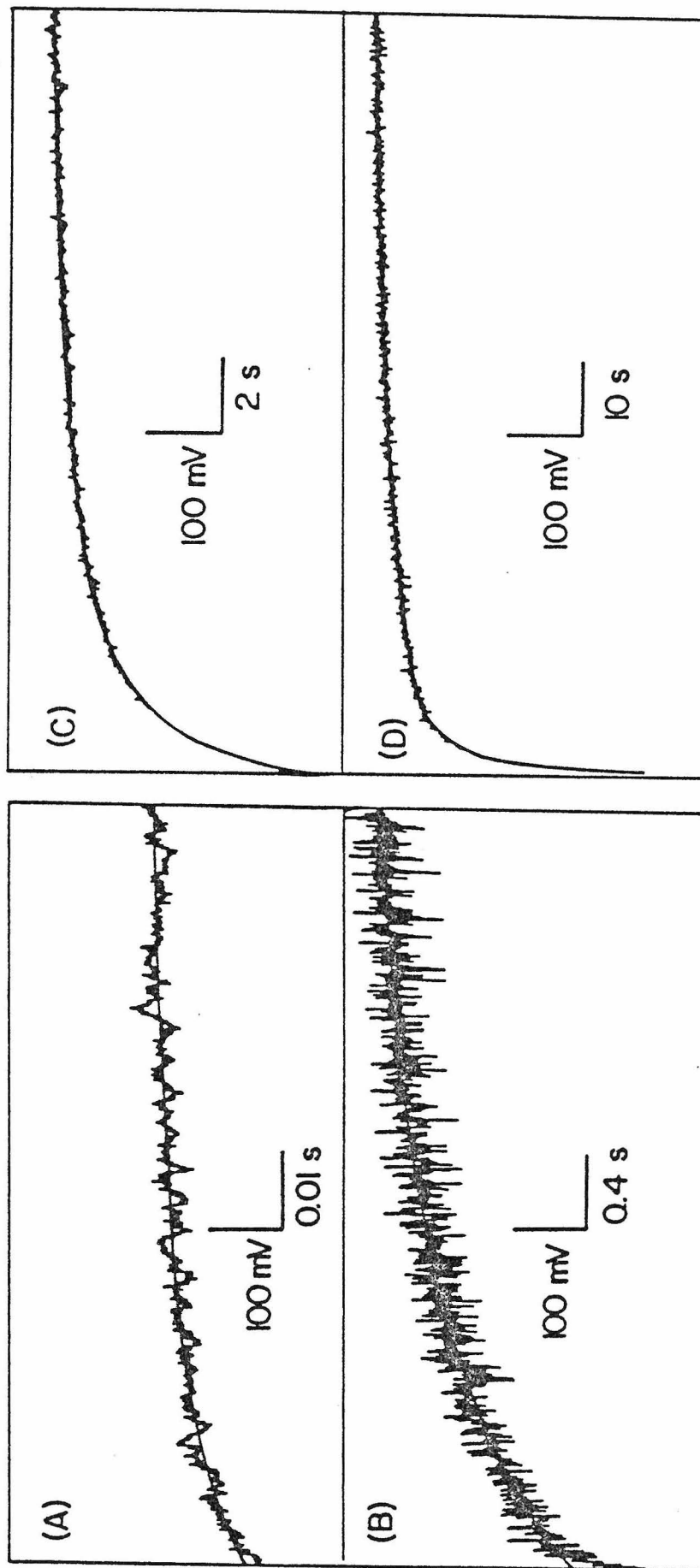
FIGURE 5: Kinetics of Carb Binding to IAS-Labeled Membrane Fragments: Stopped-flow traces were obtained by rapid mixing of 0.5 μ M AcChR (in α -BuTx sites) and 7.5 μ M Carb (final concentrations after mixing). The solid lines were calculated from the kinetic parameters given below which were obtained from non-linear regression fitting of the data to either a single exponential or to a sum of two exponentials as described in Methods. Parameters given in parenthesis were not sufficiently reliable for use in further analysis (see Text).

(A) Fastest Phase (Phase 1): $A_1 = 91$ mV; $k_1 = 54.0$ s⁻¹.

(B) Fast Time Scale: $A_1 = 206$ mV; $k_1 = 0.96$ s⁻¹.

(C) Intermediate Time Scale: ($A_1 = 173$ mV); ($k_1 = 1.25$ s⁻¹);
 $A_2 = 135$ mV; $k_2 = 0.29$ s⁻¹.

(D) Slow Time Scale: ($A_1 = 266$ mV); ($k_1 = 0.69$ s⁻¹); $A_2 = 73$ mV;
 $k_2 = 0.076$ s⁻¹.



scale of the experiment exceeded the half-time of that phase by a factor of less than 2.5 or more than 25 were rejected. In this way reliable data could be obtained for each of the three slower phases over a wide range of ligand concentrations.

The kinetic behavior of Carb binding to several membrane preparations has been examined and little preparation dependence has been observed. In addition neither the age of the preparation nor the exposure of the membrane fragments to calcium during the course of an experiment noticeably perturbed the observed kinetics (cf. Quast et al., 1979).

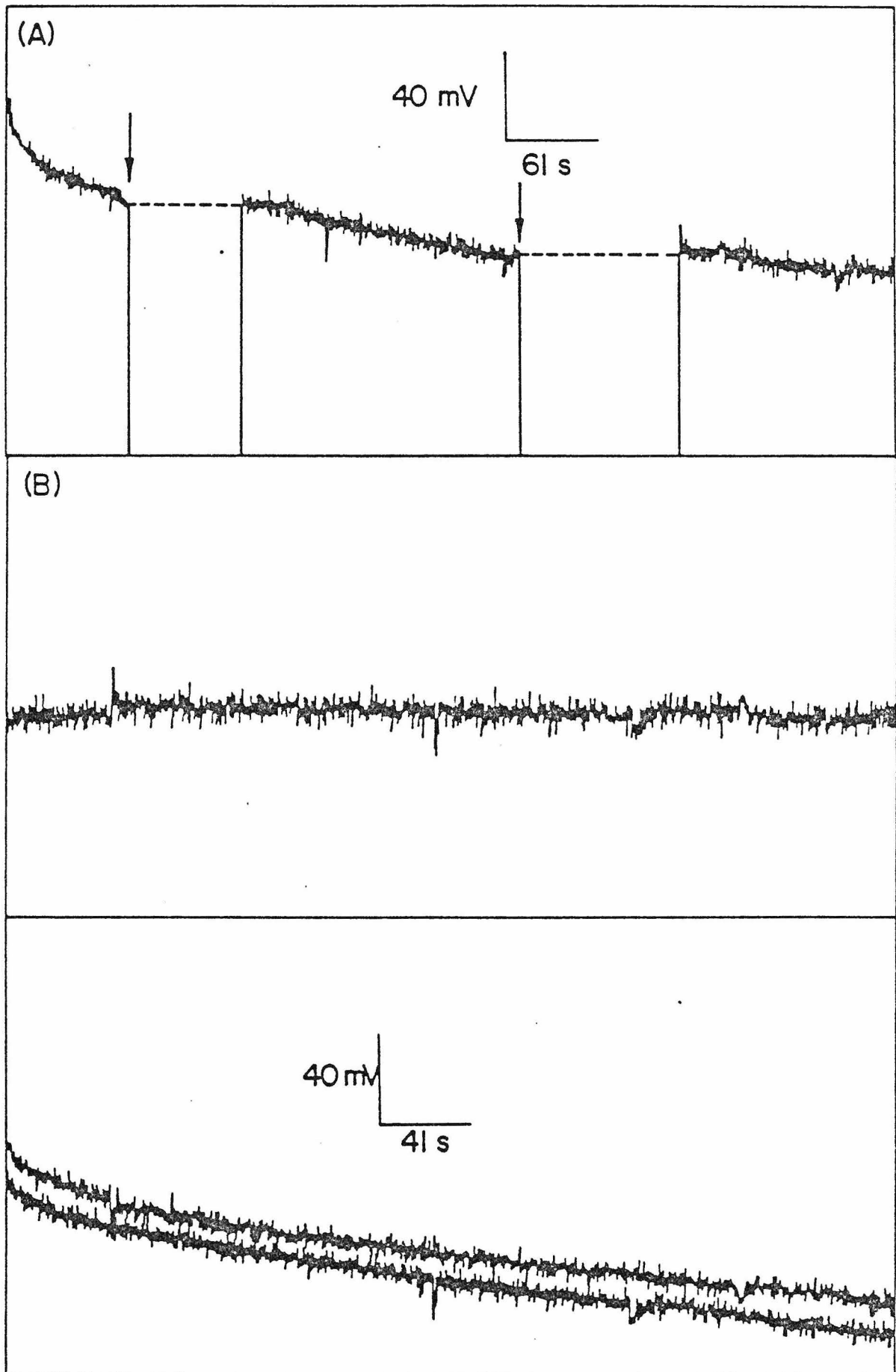
(5) Specificity of the Fluorescence Signal Change:

When α -BuTx was mixed with IAS-labeled membrane fragments the major component of the reaction trace was a linear decrease in fluorescence. The experiment illustrated in Fig. 6A showed that this decrease was due to photolytic reactions. At the points marked the excitation slit was closed thus shielding the reaction mixture from the light source. When the slit was reopened after a short time interval the fluorescence level returned to that prior to slit closure. This showed that the signal decrease occurred only under illumination and was therefore due to photolysis. The presence of α -BuTx appeared to increase the rate of photolysis, possibly due to an increase in absorbance of the probe as a result of energy transfer from the toxin. In the absence of α -BuTx, the light dependent effects were much reduced and when membrane fragments were mixed with Ringers alone there was no evidence of the nonlinearity shown in Fig. 6A which

FIGURE 6: Effect of α -BuTx on Fluorescence of IAS-Labeled Membrane

Fragments: (A) Kinetic trace recorded when $2\text{ }\mu\text{M}$ α -BuTx was mixed with $0.5\text{ }\mu\text{M}$ AcChR (final concentrations). At the points marked with an arrow the excitation slit was closed thus shielding the reaction mixture from the light source. After a short period ($\sim 1\text{ min}$) the slit was opened and signal recording was resumed. The decrease in fluorescence occurred only under illumination and was therefore due to photolysis.

(B) The lower panel shows the change in fluorescence when $0.5\text{ }\mu\text{M}$ labeled AcChR which had been pre-equilibrated with $1.0\text{ }\mu\text{M}$ α -BuTx for 60 min was mixed with Ringers solution (upper trace) or with $10\text{ }\mu\text{M}$ Carb (lower trace). The difference between these traces is illustrated in the upper panel of this figure and shows that preincubation of the receptor with α -BuTx completely abolished the Carb induced fluorescence enhancement.



was observed immediately after mixing.

Figure 6B shows the kinetic traces recorded when IAS labeled membrane fragments which had been pre-equilibrated with excess α -BuTx were rapidly mixed with Ringers and with 10 μ M Carb. The difference between these two curves is plotted in the upper panel of this Figure and shows that the Carb induced fluorescence enhancement was completely blocked by prior binding of α -BuTx to the receptor. Similar results were obtained over all time scales showing that the signal observed on binding of Carb was due to IAS specifically bound to the AcChR molecule.

(6) Effect of Carb Concentration on Binding Kinetics:

The fastest phase observed (Fig. 5A) was apparent only at low ligand concentration and its rate ($30\text{--}40\text{ s}^{-1}$) showed little concentration dependence. However its amplitude, after an initial increase, began to decrease at concentrations of Carb above 2 μ M. This phase could therefore be examined over only a small concentration range. As a result, reliable estimates for the kinetic parameters of this phase are difficult to obtain. For this reason, this phase has not, at this stage, been included in the mechanism proposed in the next section. This will be further dealt with in the Discussion.

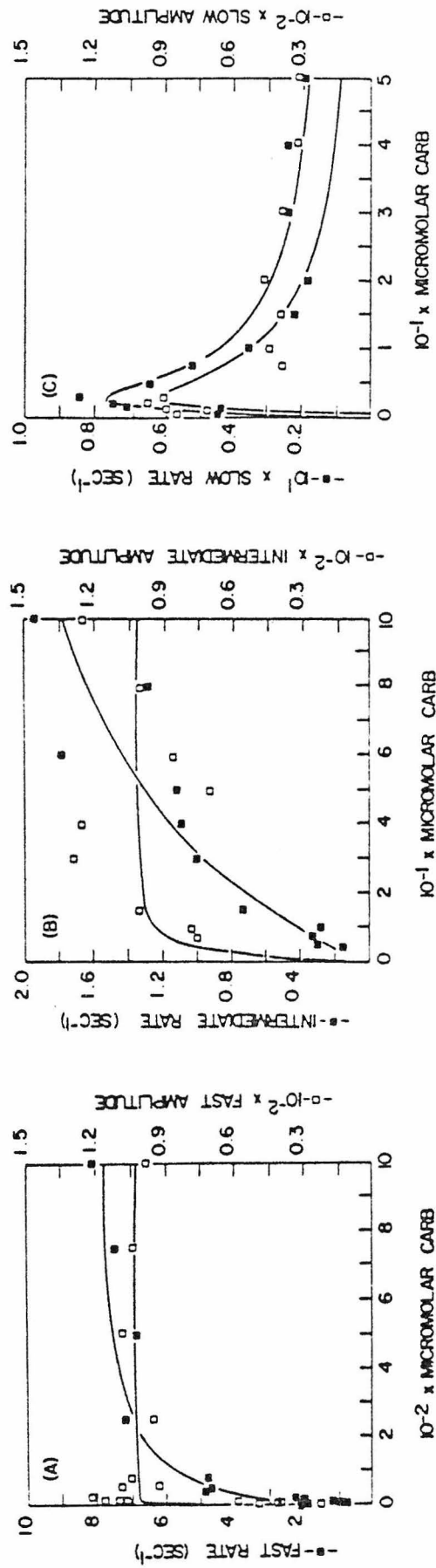
Figure 7 shows the effect of Carb concentration on the rates and amplitudes of the other three phases. The rates of both the fast (Fig. 7A) and intermediate phase (Fig. 7B) increased with increasing ligand concentration until reaching a saturating level. The kinetics of the slow phase (Fig. 7C) were apparently more complicated in that

FIGURE 7: Effect of Carb Concentration on Kinetics of Binding to IAS Labeled AcChR: The rates (■) and amplitudes (□) of the fast, intermediate and slow phases are plotted as a function of Carb concentration. Solid lines are those obtained from a fit of the data to the appropriate equations describing Mechanism 1 (see text). The kinetic parameters obtained for each phase are given below and in Table 1.

(A) Fast Phase: Rate: $k_{-2} K_4 = 46.6 \mu\text{M s}^{-1}$; $K_4 = 50.8 \mu\text{M}$, $k_2 = 8.1 \text{ s}^{-1}$
 $K_1 = 50.8 \mu\text{M}$. Amplitude: $QR_0 = 102$ (not fitted)

(B) Intermediate Phase: Rate: $k_{-5} = 0.044 \text{ s}^{-1}$; $K_1 K_2 = 5.74 \mu\text{M}$;
 $K_1 K_2 K_4 = 291.6 \mu\text{M}^2$; $k_5 / K_1 K_2 K_4 = 0.0092 \mu\text{M}^{-2} \text{ s}^{-1}$. Amplitude: $QR_0 = 104$.

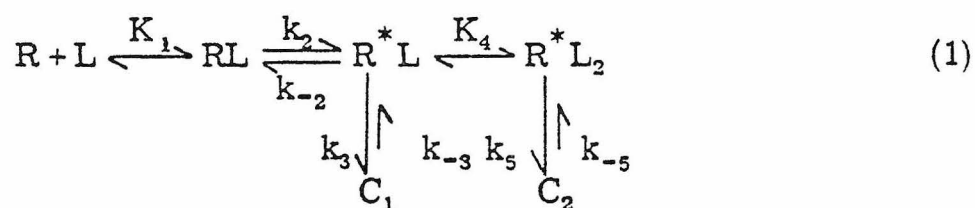
(C) Slow Phase: Rate: $k_{-3} = 0.008 \text{ s}^{-1}$, $k_3 / K_1 K_2 = 0.0723 \mu\text{M}^{-1} \text{ s}^{-1}$;
 $K_1 K_2 K_4 K_5 = 5.05 \mu\text{M}^2$. Amplitude: $QR_0 = 85$.



its rate and amplitude decreased after reaching a maximum level at approximately 2-3 μM .

(7) Kinetic Mechanism for Carb Binding:

The ligand concentration dependence of the kinetic parameters describing the intermediate and slow phases bear a striking similarity to the two phase observed when Carb binding to AcChR-enriched membranes were monitored by changes in the fluorescence of the non-covalently bound probe, ethidium (Quast et al., 1978; 1979). As was discussed by these authors, there are few simple mechanisms which can account for the observed kinetic behavior. However, one mechanism which is consistent with the ligand concentration dependencies of all three phases (omitting the fastest phase as described above) is as follows:



in which K_1 and K_4 are dissociation constants of the two receptor ligand complexes, RL and $\text{R}^* \text{L}_2$, and can be considered as fast pre-equilibria to the subsequent isomerization reactions. This mechanism is clearly very similar to that proposed by Quast et al. (1978, 1979) but is complicated by the inclusion of Step 2 ($\text{RL} \rightleftharpoons \text{R}^* \text{L}$) which corresponds to the fast phase observed. In this mechanism the intermediate phase arises from C_2 formation and the slow phase from

formation of C_1 . According to this scheme the binding of a second ligand to R^*L at high ligand concentrations drives the equilibrium in favor of C_2 formation and therefore in this concentration range the slow phase due to Step 3 is reduced.

The effect of ligand concentration on the kinetic parameters of each phase can be calculated by an extension of the procedure described by Quast et al. (1979) which is summarized in the Appendix. The proposed mechanism gives rise to three relaxation times which are designated as λ for each phase and show the following ligand concentration dependencies:

(a) Fast Phase (Fig. 7A)

$$\lambda_F = \frac{k_{-2} K_4}{K_4 + L} + \frac{k_2 L}{K_1 + L} \quad (2)$$

(b) Intermediate Phase (Fig. 7B)

$$\lambda_I = k_{-5} + \frac{k_5 \frac{L^2}{K_1 K_2 K_4}}{1 + \frac{L}{K_1 K_2} + \frac{L^2}{K_1 K_2 K_4}} \quad (3)$$

(c) Slow Phase (Fig. 7C)

$$\lambda_S = k_{-3} + \frac{k_3 \frac{L}{K_1 K_2}}{1 + \frac{L}{K_1 K_2} + \frac{L^2}{K_1 K_2 K_4 K_5}} \quad (4)$$

The rate of the slow phase reaches maximum value at a ligand concen-

tration, $L_{\max} = \sqrt{K_1 K_2 K_4 K_5}$.

Non-linear regression procedures without constraint were used to fit the rates of each phase to the derived equations. The best fit parameters obtained from different phases showed a high level of consistency and an iterative procedure rapidly converged to a set of parameters which described the behavior of each phase with precision. The kinetic constants thus obtained are given in Table I where they are compared with the corresponding values obtained by Quast *et al.* (1979).

The amplitude data were also consistent with mechanism 1 although these data themselves showed considerable scatter due to difficulties inherent in obtaining accurate amplitude data when three phases co-exist. The experimental data require that a quantum yield change occurs in each of the three isomerization steps. Furthermore, the maximum amplitudes of the three phase are similar suggesting that the three quantum yield changes are equal, a suggestion borne out by data fitting to the proposed model.

At equilibrium the total amplitude is approximately equal to the concentrations of C_1 and C_2 since the concentrations of all other species will be small. The total amplitude can therefore be approximated by the relationship

$$A_T = 2QR_0 \left[\frac{\frac{L}{K_1 K_2 K_3} + \frac{L^2}{K_1 K_2 K_4 K_5}}{1 + \frac{L}{K_1 K_2 K_3} + \frac{L^2}{K_1 K_2 K_4 K_5}} \right] \quad (5)$$

(neglecting the terms $L/K_1 K_2$ and $L/K_1 K_2 K_4$ in the denominator since

TABLE 1. Kinetic parameters for carb binding to IAS-labeled AcChR and comparison with data of Quast *et al.* (1979).

(A) Parameters Derived from Mechanism 1			(B) Data from Table 1, Row 1 of Quast <i>et al.</i> (1979)		
PARAMETER			CORRESPONDING PARAMETER		
K_1	50.8	μM	-		
k_2	8.1	s^{-1}	-		
k_{-2}	0.9	s^{-1}	-		
K_4	50.8	μM	K_4	58.0	μM
k_5	2.7	s^{-1}	k'_2	2.3	s^{-1}
k_{-5}	0.04	s^{-1}	k'_{-2}	0.10	s^{-1}
k_3	0.42	s^{-1}	k'_1	0.03	s^{-1}
k_{-3}	0.008	s^{-1}	k'_{-1}	0.002	s^{-1}
K_1K_2	5.7	μM	K_1	1.3	μM
$K_1K_2K_3$	0.12	μM	$K_1K'_1$	0.10	μM
K_4K_5/K_3	42.0	μM	K_{eff}	30.0	μM
$K_1K_2K_4K_5$	5.0	μM^2	$K_1K_2K'_2$	2.6	μM^2

these are small) in which Q is the quantum yield change. The factor of 2 is introduced because of the signal change occurring in the fast phase (i.e., formation of R^*L) which is a prerequisite for both C_1 and C_2 formation.

The concentration dependence of the intermediate and slow phases are discussed in the Appendix and can be approximated by:

$$A_I = Q \left(\frac{\lambda_S}{k_{-5}} \bar{C}_1 + \frac{\lambda_S}{k_{-3}} \bar{C}_2 \right) \quad (6)$$

for the intermediate phase and

$$A_S \simeq Q \left[\left(1 - \frac{\lambda_S}{k_{-5}} \right) \bar{C}_1 + \left(1 - \frac{\lambda_S}{k_{-3}} \right) \bar{C}_2 \right] \quad (7)$$

for the slow phase (bars denote concentrations at equilibrium).

The fits of the data to these equations are shown in Fig. 7. The values obtained for Q from independent fitting of the intermediate and slow phase data were 104 and 85 (arbitrary units) respectively which are in good agreement with the estimate of 109 obtained from the total amplitude (data not shown). The fast phase was not fitted directly (Fig. 7A) but could not be fitted with precision using the values obtained from the other three fits.

Discussion

A reactive disulphide bond near an agonist binding site of the nicotinic AcChR (Karlin, 1969) can be readily reduced and alkylated by certain affinity reagents both antagonists (Weill et al., 1974) or an agonist (Moore and Raftery, 1979a). This suggested its usefulness as a target for alkylation by a sulphydryl-specific reagent whose fluorescence could be used to monitor the interactions of agonists and the receptor molecule. IAS has proved to be an ideal probe for this purpose since its ready solubility in water reduces the possibility of non-covalent interaction with membrane lipids and facilitates the alkylation reaction. More importantly, the fluorescence of the probe is sensitive to environmental changes and because of its small size it does not appear to seriously perturb the agonist binding properties of the receptor.

Equilibrium binding assays using radioactive Carb have shown that for most preparations neither the K_d of the complex nor the stoichiometry of Carb and toxin binding was altered as a result of IAS modification. In these preparations a change to a state having high affinity for agonists could be induced by exposure to Carb leading to an overall K_d for Carb of approximately 100 nM. In a few experiments however a slight decrease in the apparent number of Carb sites was found after treatment. Moore and Raftery (1979b) have reported that reduction of the membrane bound receptor with 1 mM DTT followed by iodoacetamide alkylation resulted in a blocking of the receptor in a form having low affinity for agonists. Such membrane

preparations have a K_d for Carb binding close to 30 μM . A possible explanation for the slight loss of binding sites during IAS modification is therefore that in spite of the low DTT concentration used (50 μM) partial blocking of the receptor in the low affinity form had occurred. Under the experimental conditions of the binding assays, binding of Carb to these low affinity sites would not be apparent and thus no biphasicity of the Scatchard plots would be expected.

Reaction of AcChR with IAS inhibited the covalent labeling of the receptor by the alkylating agonist bromoacetylcholine. This strongly suggests that the modified disulphide bond which is responsible for the fluorescence enhancement induced by the binding of Carb is located near the agonist binding site. Reversible binding of bromoacetylcholine was not noticeably affected by receptor modification and this is further evidence that agonist and IAS can co-exist at the binding site.

The fluorescence properties of bound IAS have been shown to be sensitive to the interaction of Carb and the receptor. In addition the signal change observed was specific since it was completely abolished by preincubation of the receptor with α -BuTx. The consistency of the K_d values obtained from radioactive ligand bindings assays (Fig. 3) and from fluorescence titration data (Fig. 4) showed that the Carb induced fluorescence enhancement is an accurate measure of complex formation.

The fluorescence enhancement accompanying Carb binding to IAS labeled membrane fragments could be resolved into four distinct components each of which could be fitted with precision to a single exponential (Fig. 5). The rates of the three slower phases were

sufficiently well separated that reliable measurements of their kinetic parameters were obtained over a wide range of ligand concentrations (Fig. 7). A model (mechanism (1)) has been proposed which is consistent with the behavior of these phases. This mechanism is necessarily complex and includes two ligand binding steps and three conformational changes each of which is accompanied by a change in the quantum yield of bound IAS. The overall equilibrium constant for the binding of the first ligand derived for this mechanism ($K_1K_2K_3$) is 116 nM (Table 1) which is an excellent agreement with that of 93 nM measured by centrifugation assay of Carb binding to the high affinity site (Fig. 3). An effective dissociation constant for the second ligand binding can be approximated by K_4K_5/K_3 (see Quast et al., 1979) giving a value of 42 μ M (Table 1). Thus there is a significant difference in the affinities of the two binding sites and, as a consequence of the experimental conditions used, binding of this second ligand would not be expected to contribute to the equilibrium binding assays shown in Figs. 3 and 4.

The similarity of the mechanism discussed here and that proposed by Quast et al. (1978, 1979) to account for agonist binding behavior observed by changes in ethidium fluorescence is remarkable. Undoubtedly both IAS and ethidium to some extent themselves perturb the system under study. However, the finding that two entirely different approaches indicate agonist binding behavior which is consistent with the same general mechanism is clearly important.

The major difference between the two mechanisms is that here the appearance of the fast phase necessitates the inclusion of an

additional isomerization step immediately following the binding of the first ligand (Step 2; mechanism (1)). Omission of this phase would not lead to a degeneration of mechanism (1) to that proposed by Quast *et al.* (1978, 1979). In the absence of the fast step the equilibrium constant for the first ligand binding would be given by K_1K_3 which has a value of $1 \mu\text{M}$ (from Table 1). This is clearly inconsistent with both the equilibrium binding data presented here and the value of $0.10 \mu\text{M}$ reported by Quast *et al.* (1979; Table 1). Mechanism (1) should not therefore be considered as an extension of the previously proposed model but rather as a more complete form of that mechanism. It is possible that when ethidium was used to monitor agonist binding kinetics, Step 2 of mechanism 1 was a mute step in that no change in ethidium fluorescence occurred in the formation of R^*L . The binding of the first ligand and isomerization of the initial complex to R^*L would then approximate a fast equilibrium reaction and would not therefore seriously affect the kinetic analysis of the two slower phases.

Comparison of the parameters obtained from fitting mechanism (1) with those reported by Quast *et al.* (1979) indicates some discrepancies in the values describing the slow phase which we find to be an order of magnitude higher than those previously reported (Table 1). It should be noted however that these constants affect only the magnitude of the maximum rate of the slow phase occurring at low ligand concentration (Fig. 7C) and thus can be varied without substantial effect on the mechanism. A more significant variation in the kinetics observed in the present study from those described by Quast *et al.* (1978, 1979)

is that at high ligand concentration we see no evidence for the binding of additional ligand molecules. Despite these differences the similarity of the kinetic behavior observed by these two independent approaches is perhaps surprising since the membrane preparations used here consistently showed a stoichiometry of one high affinity Carb site per α -BuTx site whereas the preparations used by Quast *et al.* (1979) had a ratio of Carb to toxin sites of 0.5. A possible interpretation for this is that only one of the two Carb sites contains a disulphide bond capable of modification by IAS. In such a case, the fluorescence of bound IAS would reflect the kinetics of Carb binding to only half of the available sites. However, the linearity of the Scatchard plots shown in Fig. 3 indicates one homogeneous class of non-interacting Carb binding sites. It would therefore be possible that even though only half of the binding sites had been labeled by IAS the signal change observed on binding of Carb would be an accurate reflection of the behavior of the receptor as a whole.

Despite its complexity, mechanism (1) cannot account for the appearance of a faster phase (Fig. 5A) which was observed at low ligand concentration and had a rate of $30\text{--}40\text{ s}^{-1}$. As was described in the experimental section this phase was necessarily neglected in the discussion of the proposed mechanism because of the unreliability of the kinetic constants obtained for this phase. As a consequence of this omission, mechanism (1) must be considered as only a simplified model for Carb binding. It is clear from the results that phase 1 cannot be attributed to a ligand binding step since its rate does not

significantly increase with ligand concentration as would be expected of a bimolecular reaction. In addition the amplitude of this phase seems to parallel that of the slow phase and this leads to the tentative proposal that these two phases are closely related. One possibility is that Phase 1 arises from formation of an intermediate in the $R^*L \rightleftharpoons C_1$ conformational change. Such a scheme is qualitatively consistent with the data and although it changes the interpretation of some kinetic parameters it does not affect the form of the concentration dependence of the other phases. However, assignment of this phase must await more reliable evidence which may be obtained from studies of other cholinergic effectors.

Heidmann and Changeux (1979) have recently reported their results from a study of the kinetics of binding of a fluorescent agonist (Dansyl- C_6 -choline) to membrane bound AcChR from Torpedo marmorata. They have interpreted their results in terms of a model in which there are two pre-existing states of the receptor which are interconvertible. The observed kinetics of Carb binding to IAS-labeled membrane fragments are quite different from those seen for Dansyl- C_6 -choline binding and are inconsistent with such a two-state mechanism.

In the absence of supporting evidence it is difficult to correlate the kinetic behavior described here with the physiological responses of the receptor. However, the results require that at least two ligands must bind to the IAS-labeled receptor which is consistent with electrophysiological studies which indicate that more than a single ligand binding is required to activate the ion channel in vivo. No attempt has

been made here to ascribe any step to the opening of the channel in the membrane. It is hoped that further information obtained from examination of the effects of channel blockers such as histrionicotoxin or local anesthetics on agonist binding kinetics will aid in the interpretation of the kinetic data at the functional level of the receptor.

Appendix

The mathematical treatment of mechanism (1) given below is an extension of that given by Quast et al. (1979) to include the fast relaxation, Step 2.

Rates

The fluorescence signal, F , at time t is proportional to the formation of $Y + C_1 + C_2$ where Y is equal to $R^*L + R^*L_2$. From mechanism 1 the rates of change of the concentrations of these three species are given by the rate equations:

$$\begin{aligned}\dot{Y} &= k_2RL - (k_{-2} + k_3) R^*L + k_{-3} C_1 + k_{-5} C_2 - k_5 R^*L_2 \\ \dot{C}_1 &= k_3R^*L - k_{-3} C_1 \\ \dot{C}_2 &= k_5R^*L_2 - k_{-5} C_2\end{aligned}\tag{A1}$$

where '.' indicates the derivative with respect to time. Assuming that the ligand binding steps are fast and can be considered as pre-equilibrium reactions to the isomerization steps and using the mass balance $R_0 = R + RL + Y + C_1 + C_2$ the rate equation can be rewritten, in matrix form as:

$$\begin{bmatrix} \dot{C}_1 \\ \dot{C}_2 \\ \dot{Y} \end{bmatrix} + A \begin{bmatrix} C_1 \\ C_2 \\ Y \end{bmatrix} = \begin{bmatrix} 0 \\ 0 \\ \alpha R_0 \end{bmatrix}\tag{A_2}$$

where A is the reaction matrix

$$\begin{bmatrix} k_{-3} & 0 & -\gamma \\ 0 & k_{-5} & -\delta \\ \alpha - k_{-3} & \alpha - k_{-5} & \alpha + \beta + \delta + \gamma \end{bmatrix} \quad (\text{A3})$$

and

$$\begin{aligned} \alpha &= \frac{k_2 L}{K_1 + L} & \beta &= \frac{k_{-2} K_4}{K_4 + L} \\ \gamma &= \frac{k_3 K_4}{K_4 + L} & \delta &= \frac{k_5 L}{K_4 + L} \end{aligned} \quad (\text{A4})$$

The calculation of the relaxation rates is simplified by the experimental fact that the three phases are kinetically well separated, i.e., $\lambda_F > \lambda_I > \lambda_S$. The characteristic polynomial ($|A - \lambda I| = 0$) for matrix A can be written in simple form as:

$$a_0 + a_1 \lambda + a_2 \lambda^2 + \lambda^3 = 0 \quad (\text{A5})$$

where the coefficients, a_i , are rather complicated functions of the elements of the reaction matrix, A.

Assuming that the phases are all well separated the three relaxation rates may be approximated by (cf. Quast et al., 1979):

$$\lambda_F = a_2; \quad \lambda_I = -\frac{a_1}{a_2}; \quad \lambda_S = \frac{a_0}{a_1} \quad (\text{A6})$$

These equations can be simplified by neglecting the terms which are relatively small in each equation and give the following equations:

(1) Fast Phase

$$\lambda_F \approx \frac{k_2 L}{K_1 + L} + \frac{k_{-2} K_4}{K_4 + L} \quad (\text{A7})$$

(2) Intermediate Phase

$$\lambda_I \approx k_{-5} + \frac{k_5 \frac{L^2}{K_1 K_2 K_4}}{1 + \frac{L}{K_1 K_2} + \frac{L^2}{K_1 K_2 K_4}} \quad (\text{A8})$$

(3) Slow Phase

$$\lambda_S \approx k_{-3} + \frac{k_3 \frac{L}{K_1 K_2}}{1 + \frac{L}{K_1 K_2} + \frac{L^2}{K_1 K_2 K_4 K_5}} \quad (\text{A9})$$

Amplitudes

Analysis of the amplitude data is simplified by the fact that the quantum yield change occurring in each isomerization is about equal. As described in the text the total amplitude is approximated by the relationship:

$$A_T = 2Q(\bar{C}_1 + \bar{C}_2) \quad (\text{A10})$$

the factor of 2 being introduced as a result of the fluorescence change occurring in the formation of R^*L .

The fluorescence signal at time t , $F(t)$ is proportional to the

concentration of $Y + C_1 + C_2$:

$$F(t) = A_T - A_F \exp(-\lambda_F t) - A_I \exp(-\lambda_I t) - A_S \exp(-\lambda_S t) \quad (A11)$$

The amplitude of each phase can be calculated using Eq. (A1) from the boundary conditions:

$$\begin{aligned} F(0) &= 0 = A_T - A_F - A_I - A_S \\ \dot{F}(0) &= 0 = A_F \lambda_F + A_I \lambda_I + A_S \lambda_S \\ \ddot{F}(0) &= QR_0 \alpha(\gamma + \delta) = A_1 \lambda_F^2 + A_2 \lambda_I^2 + A_3 \lambda_S^2 \end{aligned} \quad (A12)$$

This can be rewritten in matrix notation:

$$\begin{bmatrix} -1 & -1 & -1 \\ \lambda_F & \lambda_I & \lambda_S \\ \lambda_F^2 & \lambda_I^2 & \lambda_S^2 \end{bmatrix} \begin{bmatrix} A_F \\ A_I \\ A_S \end{bmatrix} = \begin{bmatrix} A_T \\ 0 \\ QR_0 \end{bmatrix} \quad (A13)$$

Neglecting small terms of the order of λ_S^2 one obtains:

$$A_I = Q \frac{\lambda_S}{k_{-5}} \bar{C}_1 + Q \frac{\lambda_S}{k_{-3}} \bar{C}_2 \quad (A14)$$

for the intermediate amplitude and

$$A_S = Q \left[\left(1 - \frac{\lambda_S}{k_{-5}}\right) \bar{C}_1 + \left(1 - \frac{\lambda_S}{k_{-3}}\right) \bar{C}_2 \right] \quad (A15)$$

for the slow amplitude. The two equations (A14) and (A15) are identical to those derived by Quast et al. (1979) which is not unexpected

since for those calculations the fast phase (Step 2) can be considered to approximate a fast pre-equilibrium to the formation of C_1 and C_2 .

The fast phase amplitude can be obtained from the relationship:

$$A_F = A_T - A_I - A_S = Q[\bar{C}_1 + \bar{C}_2]. \quad (A16)$$

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